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Original Research Article

Associations between *NOD2*, *IRGM* and *ORMDL3* polymorphisms and pediatric-onset inflammatory bowel disease in the Lithuanian population

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ABSTRACT

Background and objective: Recent GWAS and meta-analyses have revealed about 200 susceptibility genes/loci for inflammatory bowel diseases (IBD). However, only a small number of studies were performed in early-onset IBD. The aim of this study was to assess the association between NOD2, IL23R, ATG16L1, IRGM, IL10, NKX2-3 and ORMDL3 variants and early-onset IBD.
Materials and methods: A total of 76 affected individuals (30 with Crohn's disease [CD] and 46 with ulcerative colitis [UC]) at the age of ≤17 years and 158 matched controls recruited in Lithuania were genotyped for the known genetic susceptibility variants in NOD2 (Arg702Trp (rs2066844), Gly908Arg (rs2066845) and Leu1007insC (rs2066847)), IL23R (rs11209026), ATG16L1 (rs2241880), IRGM (rs4958847), IL10 (rs3024505), NKX2-3 (rs11190140) and ORMDL3 (rs2872507) genes.
Results: Variants in NOD2 (Leu1007insC) and IRGM genes increased risk for CD (OR = 6.56, 95% CI: 2.54–16.91, $P = 1.21 \times 10^{-5}$ and OR = 2.32, 95% CI: 1.05–5.14, $P = 0.033$; respectively); whereas a variant in ORMDL3 gene was strongly associated with UC (OR = 1.99, 95% CI: 1.23–3.20, $P = 4.15 \times 10^{-3}$).
Conclusions: The results confirmed that polymorphisms in NOD2 (Leu1007insC) and IRGM genes are associated with increased risk of CD; whereas the ORMDL3 variant is associated with susceptibility to UC in the Lithuanian early-onset IBD population.

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1. Introduction

Inflammatory bowel disease (IBD) such as Crohn's disease (CD) and ulcerative colitis (UC) are common inflammatory conditions of the gastrointestinal tract. Early-onset IBD, however, has unique characteristics of phenotype, severity and familiarity [1]. Only limited data are available for pediatric IBD epidemiology in Eastern Europe, including the Baltic countries. EpiCom study data suggest that the incidence rates of IBD are increasing in the adult population (6.6 cases per 10 000 for UC and 2.6 cases per 10 000 for CD) [2]; however, there are no data on the incidence of children IBD in Lithuania. According to retrospective study data from the Hospital of Lithuanian University of Health Sciences (50 000 of children visits yearly), the incidence rate of pediatric IBD is increasing as well: from 2 new cases in 2000 to 10 new IBD cases in 2012 [3].

The etiology of IBD is complex and involves both genetic and environmental triggers, including defects in bacterial clearance, defective mucosal barrier and perpetuated mucosal immune response to commensal intestinal bacteria [4].

In spite of the progress made in understanding the genetic basis of adult IBD [5], there is limited information on the genetic susceptibility of pediatric IBD. A number of studies performed in early-onset IBD patients identified adult-susceptibility loci to be significantly associated with pediatric IBD (23 loci in common), including gene variants attributed to the recognition of bacterial products (*NOD2*), loci coding for cytokines involved in innate and adaptive immune responses (*IL23R*, *IL10*), genes related to the autophagy pathway (*ATG16L1*, *IRGM*) and sequence variations in genes encoding for basic developmental functions (*NKX2-3*) [6]. These loci have been replicated in a number of independent pediatric-onset IBD studies [7,8].

In this study we investigated whether potential variants in *NOD2*, *IL23R*, *ATG16L1*, *IRGM*, *IL10*, *NKX2-3* and *ORMDL3* genes, reported in the previous meta-analysis and GWA studies are associated with pediatric IBD in a low-incidence population of Lithuania.

2. Materials and methods

2.1. Patients and controls

The study consisted of 76 affected individuals, including 30 with CD and 46 with UC, and healthy, age- and gender-matched controls ($n = 158$). All individuals were diagnosed under the age of 17 years and fulfilled standard IBD diagnostic criteria [9] and phenotype characterization based on the Montreal classification [10]. Consecutive IBD patients were recruited in the Department of Pediatrics, Hospital of Lithuanian University of Health Sciences and Panevėžys District Hospital during 2003–2009. Children with functional dyspepsia and without organic diseases of the digestive tract and liver were included in the control group. All study participants were of Caucasian ethnicity. The full demographic and phenotypic descriptions of the study group are summarized in Table 1.

2.2. Genotyping

The two *NOD2* variants – Arg702Trp (rs2066844 [ID C__11717468_20]), Gly908Arg (rs2066845 [ID C__11717466_20]), *IL23R* variant Arg381Gln (rs11209026 [ID C___1272298_10]), ATG16L1 variant Thr300Ala (rs2241880 [ID C___9095577_20]), IRGM (rs4958847 [ID C___1398968_10]) – and *IL10* variant (rs3024505 [ID C__15983681_20]) were genotyped using pre-designed TaqMan® single nucleotide polymorphism (SNP) genotyping assays. The *NOD2* variant Leu1007insC (rs2066847) was genotyped using Custom TaqMan® SNP Genotyping Assay (Life Technologies, USA) [11]. Genotyping was performed using TaqMan® technology from Life Technologies (formerly Applied Biosystems, CA, USA) according to the manufacturer's recommendations. Genotype assignments were manually confirmed by visual inspection with 7500 software v 2.0.5 compatible with the TaqMan® system.

2.3. Statistical analysis

Quality assessments and statistical analysis of the genotyping data were performed using PLINK software version 1.07 [12]. Individuals with more than 10% missing genotypes and SNPs with a call-rate below 90% or deviation from Hardy-Weinberg equilibrium (HWE) in the controls ($P < 0.05$) were excluded from further analysis. In total, 99% of all cases and controls were successfully genotyped.

Differences in allele frequencies between cases and controls were calculated using χ^2 statistics. As the SNPs under study were previously associated with pediatric IBD at genome-wide significance level ($P < 5 \times 10^{-8}$), an association level of $P < 0.05$ with the same risk allele identified in the index studies was defined.

Subphenotypes of IBD (disease localization (defined macroscopically as extensive, left-sided, or proctitis only), extra-intestinal manifestations), disease modifiers (age at diagnosis and family history of disease) and disease outcome measures (surgery and treatment using biological therapy) were inspected in within-case analyses. The χ^2 test was used to detect association between each binary phenotype and the genotyped SNPs. *P* values were adjusted for multiple comparisons based on the Bonferroni procedure (correction was applied for the number of complementary subgroups of patients).

3. Results

Genotype and allele distributions for *NOD2* (*rs2066844*), (*rs2066845*) and (rs2066847), *IL23R* (rs11209026), (rs13361189), *ATG16L1* (rs2241880), *IRGM* (rs4958847), *IL10* (rs3024505), *NKX2-3* (rs11190140) and *ORMDL3* (rs2872507) genes in the CD, UC, and control groups are presented in Tables 2 and 3, respectively. All investigated polymorphisms were in accordance to the Hardy–Weinberg equilibrium.

In this study, none of the studied individuals were carriers of all three NOD2 risk alleles. The combined *NOD2* allele carriership in the group of patients with CD was much higher than in controls (41.9% vs. 13.4%) and resulted in significant association (OR = 4.6, 95% CI: 2.00–10.92, $P < 0.001$); whereas

Table 1 – Summary of clinical characteristics of the IBD children and controls.

Characteristics	CD (n = 30)	UC (n = 46)	Controls (n = 158)
Gender (male/female), n	21/9	20/26	88/70
Age, mean ± SD, years	13.23 ± 3.5	13.17 ± 3.9	3.63 ± 3.247
Time before diagnosis, mean ± SD, months	2.86 ± 1.9	2.73 ± 3.6	–
Familial IBD	1 (3.3)	3 (6.5)	–
Disease extension in UC			
Proctitis, E1	–	14 (30.4)	–
Left-sided colitis, E2	–	9 (19.6)	–
Extensive colitis, E3	–	23 (50)	–
Disease localization in CD			
Terminal ileum, L1	8 (26.7)	–	–
Colon, L2	12 (40.0)	–	–
Ileocolon, L3	10 (33.3)	–	–
Upper GI, L4	–	–	–
Terminal ileum + upper GI, L1 + L4	–	–	–
Colon + upper GI, L2 + L4	2 (2.6)	–	–
Ileocolon + upper GI, L3 + L4	2 (2.6)	–	–
Disease behavior in CD			
Non-stricturing, non-penetrating, B1	23 (76.7)	–	–
Stricturing, B2	6 (20.0)	–	–
Penetrating, B3	1 (3.3)	–	–
Perianal disease (isolated), B4	–	–	–
Non-stricturing, non-penetrating + perianal, B1p	6 (20.0)	–	–
Stricturing + perianal, B2p	4 (13.3)	–	–
Penetrating + perianal, B3p	1 (3.3)	–	–
Extraintestinal manifestations			
Joints	5 (16.7)	2 (4.3)	–
Cutaneous	1 (3.3)	2 (2.2)	–
Ocular	–	–	–
Hepatobiliary	1 (3.3)	2 (4.3)	–
Biological therapy/no biological therapy, n	5/25	4/42	–
Surgery treatment	3 (10.0)	4 (8.7)	–

Values are number (percentage) unless otherwise indicated.
IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis.

Table 2 – Genotype and allele frequencies of *NOD2*, *ATG16L1*, *IL23R*, *IRGM*, *IL10*, *ORMDL3* and *NKX2-3* polymorphisms in the Lithuanian pediatric CD patients and controls.

Gene marker	Minor allele	CD (n = 30)		Controls (n = 158)			
		MAF	GT (11,12,22)	MAF	GT (11,12,22)	P	OR (95% CI)
NOD2							
rs2066845	C	0.016	0/1/30	0.012	0/4/154	0.826	1.27 (0.14–11.63)
rs2066847	insC	0.161	2/6/23	0.028	0/9/149	1.21×10^{-5}	6.56 (2.54–16.92)
rs2066844	T	0.080	0/5/26	0.029	0/9/146	0.051	2.93 (0.94–9.07)
ATG16L1							
rs2241880	G	0.451	7/14/10	0.503	40/78/39	0.458	0.81 (0.47–1.40)
IL23R							
rs11209026	A	0	0/0/31	0.047	2/11/145	0.080	–
rs13361189	C	0.080	0/5/26	0.044	0/14/144	0.231	1.89 (0.65–5.46)
IRGM							
rs4958847	A	0.161	0/10/21	0.076	0/24/133	0.033	2.32 (1.05–5.14)
IL10							
rs3024505	A	0.177	0/11/20	0.158	3/44/111	0.707	1.14 (0.55–2.35)
ORMDL3							
rs2872507	A	0.338	5/11/15	0.334	19/67/71	0.947	1.02 (0.57–1.81)
NKX2-3							
rs11190140	T	0.500	7/17/7	0.477	38/75/45	0.749	1.09 (0.63–1.88)

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 ($P < 0.05$), odds ratios (OR, shown for the minor allele) with 95% confidence intervals (CI) are depicted CD case–control population.

Table 3 – Genotype and allele frequencies of *NOD2*, *ATG16L1*, *IL23R*, *IRGM*, *IL10*, *ORMDL3* and *NKX2-3* polymorphisms in the Lithuanian pediatric UC patients and controls.

Gene marker	Minor allele	UC ($n = 46$)		Controls ($n = 158$)			
		MAF	GT (11,12,22)	MAF	GT (11,12,22)	*P*	OR (95% CI)
NOD2							
rs2066845	C	0	0/0/145	0.012	0/4/154	0.144	0.14 (0.01–2.23)
rs2066847	C	0.055	1/3/41	0.028	0/9/149	0.214	2.00 (0.65–6.14)
rs2066844	T	0.029	0/2/43	0.022	0/9/146	0.728	0.76 (0.16–3.58)
ATG16L1							
rs2241880	G	0.496	11/24/10	0.501	39/78/40	0.811	1.05 (0.66–1.69)
IL23R							
rs11209026	A	0.022	0/2/43	0.047	2/11/145	0.385	0.45 (0.10–2.03)
rs13361189	C	0.044	1/2/42	0.044	0/14/144	0.995	1.00 (0.32–3.12)
IRGM							
rs4958847	A	0.066	1/4/40	0.076	0/24/133	0.755	0.86 (0.34–2.18)
IL10							
rs3024505	A	0.155	1/12/32	0.158	3/44/111	0.951	0.98 (0.51–1.86)
ORMDL3							
rs2872507	A	0.500	13/19/13	0.334	19/67/71	4.15×10^{-3}	1.99 (1.23–3.20)
NKX2-3							
rs11190140	T	0.444	10/20/15	0.477	38/75/45	0.575	0.87 (0.54–1.40)

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 ($P < 0.05$), odds ratios (OR, shown for the minor allele) with 95% confidence intervals (CI) are depicted UC case–control population.

no statistically significant difference was detected in UC group.

Allelic single marker analysis in the early-onset CD group revealed a significant association with *NOD2* variant Leu1007insC (16% of CD patients vs. 2% of controls; OR = 6.56, 95% CI: 2.54–16.91, $P = 1.21 \times 10^{-5}$) and *IRGM* variant (rs4958847) (16% of CD patients vs. 7% of controls; OR = 2.32, 95% CI: 1.05–5.14; $P = 0.033$). In the UC group, a significant disease association with SNP in *ORMDL3* gene (rs2872507) was identified (50% of UC patients vs. 33% of controls; OR = 1.99, 95% CI: 1.23–3.20, $P = 4.15 \times 10^{-3}$).

Neither the other two *NOD2* variants nor the known variants in *IL23R*, *NKX2-3* and *ATG16L1* genes were found to be risk factors for CD, UC or IBD, as our relatively small study population was underpowered to demonstrate such weak to moderate disease associations.

3.1. *Genotype–phenotype correlations of IBD patients*

Our phenotype analysis data showed that children with IBD had impaired height (15.79% of CD cases and 6.45% of UC cases) and weight (31.58% of CD cases and 29.03% of UC cases). Further, we assessed whether the investigated SNPs could be in association with specific disease phenotypes, such as gender, disease location and behavior, surgery, family history, extra-intestinal manifestations, and medical therapies.

However, no significant associations were found for sub-phenotypes under study following correction for multiple testing.

4. Discussion

Recent GWA studies have discovered several novel genes and loci involved in the pathogenesis of IBD. Most associations appear to be common to both types of IBD, while some genes/loci may be specific to adult- or pediatric-onset, and the factors that determine age of onset are unknown at present.

This pediatric IBD case–control study, carried out with previously reported *NOD2*, *IL23R*, *ATG16L1*, *IRGM*, *IL10*, *NKX2-3* and *ORMDL3* disease associated variants, was the first in Lithuania. Limited data are available on the incidence rates of IBD, especially in children, in North-East European countries. The recent data in Lithuania have shown low but gradually rising incidence of adult IBD, however there are no data regarding children with IBD in Lithuania. Therefore, analysis of the genetic contribution to disease susceptibility in this region was of great interest.

In contrast to the wealth of published data in adults, there are only a small number of published studies looking at *NOD2* mutations exclusively in pediatric IBD populations [13]. *NOD2* gene belongs to Apaf-1/Ced-4 superfamily, which is responsible for the regulation of apoptosis, as well as to pattern recognition receptor group that participates in recognition of bacteria products, such as muramyl dipeptide, through the leucine-rich repeat (LRR) areas [14]. Today more than 60 polymorphisms of this gene have been identified; however, three common mutations (Leu1007fsinsC, Arg702Trp and Gly908Arg) have been specifically associated with ileal involvement, stricturing complications and earlier age of onset [15]. However, the heterogeneity of these mutations differs not only between ethnic groups, but also across different countries. In the present study, the carriage of at least one *NOD2* variant was highest in the CD patients group compared to the control (41.9% and 13.4%, respectively; $P < 0.001$). Moreover, our results confirmed the strong association of *NOD2* Leu1007insC mutation with disease susceptibility in CD in Lithuanian pediatric IBD. Frequency of the Leu1007insC (rs2066847) minor allele was significantly elevated among the cases (16%) vs. that among controls (2%). These findings are in contrast with previous reports from other European populations. Risk allele frequencies were reported

significantly lower in the Scottish, Sweden and Italian pediatric CD populations, for the variant Leu1007insC in cases 4.2%, 1.7% and 7.4%, respectively [16,17]. The Leu1007insC minor allele frequencies were similar to the published studies performed in USA but significantly lower to those of German pediatric CD population (17% and 25.4%, respectively) [18,19]. However, we did not confirm the association between the SNPs Arg702Trp and Gly908Arg and CD susceptibility in our study group.

Hampe et al. were the first group to implicate the autophagy pathway in IBD, especially in CD [20]. The *IRGM* gene is located on chromosome 5q33.1, encodes an autophagy-inducing protein and belongs to the immunity-related guanosine triphosphatases (IRGs). IRGs play an important role in host defense against intracellular pathogens [21]. Based on genome-wide association, a significant association between IRGM polymorphism and adult Crohn's disease was determined [22]. However, few records on IRGM association to children's Crohn's disease are given [23]. Our results provide an independent confirmation of the association between the *IRGM* (rs4958847) variant and CD. Similar genotype distributions were observed in the Italian pediatric population [24]. In contrast, other studies have shown that IRGM gene polymorphism was not associated with CD in children but only with ileal CD in the adult population [25].

Genome-wide association studies identified new UC susceptibility loci (*FCGR2A*, 5p15, 2p16, *CARD9* and *ORMDL3*). Orm 1 like 3 *ORMDL3* gene is located in chromosome 17q12, synthesized in the endoplasmic reticulum and may be involved in protein folding, and growing evidence indicates that there are interactions between the unfolded protein response (UPR) and immune responses [26]. Our study confirmed that the *ORMDL3* rs2872507 variant was associated with disease susceptibility in UC. This finding has been replicated in recently performed GWA study in a Greek population [27] Moreover, the strong association in both CD and UC was reported in the early-onset GWAS [28]. In contrast, *ORMDL3* has been suggested in adult-onset CD by GWAS meta-analysis, but not in UC [29].

Despite obvious similarities between childhood and adult onset disease there are number of differences which are unique in the pediatric IBD population. More than 35% of pediatric CD patients and up to 10% of pediatric UC patients have impaired linear growth [30]. In our study some patients with impaired weight and height were observed; however, it was not statistically significant and association with *NOD2* polymorphism was not confirmed. In contrast to other studies, we did not find any significant association between *investigated* SNPs and disease phenotype. These conflicting results can be explained by the regional and ethnic differences and relatively small number of patients included in this study.

5. Conclusions

The results of our study confirmed that polymorphisms in *NOD2* (rs2066847) and *IRGM* (rs4958847) genes are associated with increased risk of CD; whereas the *ORMDL3* (rs2872507) variant is associated with susceptibility to UC in the Lithuanian early-onset.

Conflict of interest

The authors declare no conflict of interest.

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