NOD2/CARD15 mutations in Polish and Bosnian populations with and without Crohn's disease: prevalence and genotype-phenotype analysis

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ABSTRACT

Data on prevalence and phenotypic consequences of ***nucleotide-binding oligomerisation domain 2/caspase recruitment domains 15 (NOD2/CARD15)*** variants in Crohn's disease (CD) population in Poland and Bosnia and Herzegovina (B&H) are nonexistent. We aimed to determine the prevalence of ***NOD2/CARD15*** mutations and their association with disease phenotype in Polish and Bosnian patients with CD and in healthy controls. We prospectively recruited 86 CD patients and 83 controls in Poland and 30 CD patients and 30 controls in B&H, 229 in total. We determined the prevalence of ***NOD2/CARD15*** mutations and their association with the disease phenotype according to Montreal classification. Participants were genotyped for ***Leu1007fsinsC and Gly908Arg*** mutations. At least one CD-associated allele was found in 29/86 (33.7%) of Polish CD patients and in 9/83 (10.8%) of healthy controls ($p<0.001$). In both CD patients and controls in Bosnian sample, at least one ***NOD2*** mutation was found in equal number of patients (3/30; 10%) with all of the ***NOD2*** mutation positive CD patients being homozygous, while controls being heterozygous. In Polish sample, perianal disease was less frequent in CD patients with any ***NOD2*** mutation (1/21; 4.8%) compared to those without (11/41; 26.8%; $p=0.046$). Higher percentage of patients with ***NOD2*** mutations had history of CD related surgery when compared with those without mutations (66.7% vs. 43.3%; $p=0.05$). The risk for CD is increased in patients with ***NOD2*** mutations (Poland) and especially in the presence of homozygous ***NOD2*** mutations (Poland and Bosnia). The presence of variant NOD2 alleles is associated with increased need for surgery and reduced occurrence of perianal disease.

KEY WORDS: ***NOD2/CARD15*** gene; Crohn's disease; genotype-phenotype analysis; gene frequency; Poland; Bosnia and Herzegovina
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INTRODUCTION

It is widely known that the pathogenesis of Crohn's disease (CD) is an extremely complex interplay of environmental and genetic factors [1]. Genetic factors strongly contribute to inflammatory bowel disease (IBD), especially to CD, and more than 30 distinct susceptibility gene loci for CD have been identified [2]. An important susceptibility locus in pericentromeric region of chromosome 16 is called ***IBD1***[3]. It contains a nucleotide-binding oligomerisation domain 2/caspase recruitment domains 15 (***NOD2/CARD15***) gene mutations, which are strongly associated with the increased risk of developing CD, as confirmed by several research groups [4-9]. A frame shift mutation (***SNP13: Leu1007insC***) and two single nucleotide polymorphisms (***SNP8: Arg702Trp and SNP12: Gly908Arg***) have been shown to be associated with susceptibility to CD [3, 8, 10]. It appears that around 30% of patients with CD are carriers of one copy of mutated ***NOD2/CARD15*** allele (heterozygotes) and about 17% carry two mutated alleles (homozygotes). Hetreozygotes have 2 to 4 times higher risk for developing CD, while in homozygotes this risk can be as much as 20 to 40 times higher [11].

The clinical impact of ***NOD2/CARD15*** variants is not high enough to justify the routine use of genetic tests for diagnostic purposes [1]. However, ***NOD2/CARD15*** variants have been shown to be associated with ileal location, fibrostenotic behavior and penetrating perianal disease [4, 12-17].

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Additionally, several reports suggest that *NOD2/CARD15* variants may have important role in disease behavior. Carriers appear to be associated with younger age of disease onset and there is a clear trend for a rapid and more aggressive form of CD with early and repetitive need for surgery [4, 16, 18]. This may prove to be of a prognostic value for a clinician.

Eastern Europe was traditionally considered a low incidence area for CD; however, new data confirm that incidence and prevalence are rapidly increasing in some countries, reaching moderate-to-high incidence, similar to data reported from Western European countries [19]. B&H is among countries with rapidly rising incidence and prevalence of CD and latest reports suggest that the current incidence is around 5 cases per 100 000 population [20]. Epidemiological data on CD in Poland is somewhat limited, with a report based on a single hospital-based case series of the adult patients without calculated incidence and prevalence. Yet in a later review of Polish epidemiological data on IBD, numbers from this particular study were re-analyzed and it was estimated that incidence of CD was 0.1 per 100 000 population [19, 21]. Data on the prevalence and phenotypic consequences of *NOD2/CARD15* variants in the population of Poland and B&H are nonexistent, and generally reports from Slavic countries dealing with this issue are sparse [22-24]. How high is the impact of genetic susceptibility in the pathogenesis of CD in patients in these countries is yet to be understood.

Therefore, our aim was to determine the prevalence of *NOD2/CARD15* mutations and their association with phenotypic expression of the disease, in population of Polish and Bosnian patients with CD and healthy controls.

MATERIALS AND METHODS

Participants

We conducted a multicentric study at authors' institutions in B&H and Poland. The local bioethical committees of all the participating institutions approved the protocol of the study.

We recruited 30 CD patients and 30 healthy controls in B&H. Furthermore, 86 CD patients and 83 healthy controls were recruited in Poland, for the total of 229 participants. Patients and healthy individuals for control were recruited consecutively from a pool of patients who were referred to the participating institutions for diagnostic evaluation or treatment, during the time period of 1.1.2013 to 31.07.2013. Diagnosis of CD was established according to previously defined and accepted clinical, endoscopic, radiological and histological criteria [1]. The following data were collected, either directly from CD patients or from their medical records: gender, age at diagnosis, disease duration, disease localization at diagnosis, current localization of the disease, clinical behavior, surgery and extra-intestinal manifestations. We also collected detailed history regarding the type of treatment used for CD.

Disease behavior/phenotype was assessed according to Montreal classification which is now regarded as the international standard in phenotype subtyping in CD [25]. Therefore, the age at diagnosis was coded as follows: A1 - <16 years, A2 -17-40 years, A3 - >40 years. Location of the disease was coded as: L1 – ileal, L2 – colonic, L3 – ileocolonic and L4 – isolated upper digestive. Finally, the behavior of the disease was coded as: B1 – non-stricturing, non- penetrating, B2 – stricturing, B3 – penetrating, and P - perianal disease.

Healthy individuals for the control group were selected consecutively from the pool of patients in the participating institutions. Those patients were evaluated for reasons other than CD, and have had a colonoscopy performed during their diagnostic evaluation, which enabled the exclusion of the possibility of undiagnosed CD.

Genotyping of the *NOD2/CARD15*

After giving an informed consent, each patient and healthy control underwent a venous blood sampling into the vials with EDTA. Genetic investigations were carried at Department of Gerontobiology, Pomeranian Medical University, Szczecin, Poland.

Genomic DNA was extracted from peripheral blood leukocytes using Qiagen extraction kit (Qiagen, Hilden, Germany). The extraction was performed according to the manufacturer's instructions. DNA samples were stored at 4°C until analyzed. Protocols for PCR-RFLP detection of G908R (the G>C substitution at position 2722 in the exon 8; NCBI Reference Sequence NG_2066845) and Leu1007fsinsC *NOD2/CARD15* were previously described [26]. The PCR was performed on a Hightech Thermocycler Cycler (Technology for Life, SensoQuest, Gottingen, Germany). The genotypes were determined by electrophoresis in 2% agarose gel (Sigma-Aldrich, Chemie Gmbh, Munich, Germany) stained with DNA-star dye (Lonza, Inc, Rockland, USA). Each PCR product carrying mutation was re-analyzed to verify the correctness of results. All results were found accurate.

Statistical analysis

Statistical analysis was performed using SPSS software version 20.0 (SPSS Inc., Chicago, IL, USA). Patients' characteristics are presented as mean ±SD, or median and interquartile range (IQR), as appropriate. Descriptive statistics was used for determination of baseline characteristics of groups. Between-group differences in frequencies have been investigated by using chi-squared test with Yates correction where appropriate, or by Fisher's exact test. Student t-test

was used for comparison of continuous variables. All ***p*** values were two sided, and p<0.05 was considered statistically significant.

RESULTS

We prospectively recruited a total of 229 participants, and clinical characteristics of CD patients in both countries are summarized in Table 1.

NOD2/CARD15 mutations were more frequent in Polish patients with CD compared to Polish controls (X^2=12.68; df=1; ***p***<0.001) (Table 2). Presence of any type of variant allele was associated with increased odds for CD (OR=4.18; 95%CI=1.84-9.53; ***p***<0.001).

The presence of ***NOD2 10007fs*** variant allele was also more frequently found in Polish CD patients (Fisher's test; ***p***<0.0001) with the estimated odds ratio of 7.53 (95% CI=2.74-20.69) for CD in these patients.

There was no significant difference in the presence of Gly908Arg variant allele (Fisher's test; p=0.44).

The analysis of Bosnian patients was limited with a small sample. There were no patients with ***Gly908Arg*** allele. There were three participants with ***NOD2 10007fs*** variant allele in both CD and control group, however all patients in CD group were homozygous.

Regarding the possible association between disease phenotype and the presence of ***NOD2*** mutations, it appears that in Polish group, perianal disease was less frequent in CD patients with ***NOD2*** mutation, however additional analysis failed to confirm the level of association (Fisher's test; ***p***=0.046; OR=0.14; 95%CI=0.02-1.12) (Table 3).

Additionally, significantly higher percentage of patients with ***NOD2*** mutations had history of surgical interventions due to CD when compared with those without ***NOD2*** mutations (66.7% vs. 43.3%; X2=3.73; df=1; ***p***=0.05). Other comparisons between CD patients with and without ***NOD2*** mutations were not found to be significant. This includes the ratio of patients with complicated disease behavior (B2 or B3) in patients with (10/21; 47.6%) or without ***NOD2*** mutations (18/41; 43.9%).

In Bosnian group of CD patients there was no association between NOD2 mutations and genotype, but then again, this may be attributed to the small sample size.

DISCUSSION

This study is the first report on the prevalence of ***NOD2/CARD15*** mutations in patients with CD from Poland and Bosnia and Herzegovina. Although another report from Poland also addressed this problem [27], this particular study is hampered with the fact that only one mutation (Gly908Arg) was investigated. Also, the authors did investigated association with the phenotype, but disease characteristics were not described according to Montreal/Vienna classification [27].

TABLE 1. Clinical characteristics of Crohn's disease patients in both centers

	Poland (n=62+24)*	Bosnia (n=30)
Male/female	49/37	15/15
Age (yr)	34.1±13.0	42.9±14.3
Age (montreal) (n;%)		
A1	7 (11.3)	1 (3.3)
A2	49 (79)	7 (23.3)
A3	6 (9.7)	22 (73.3)
Location (montreal) (%)		
L1	2 (3.2)	12 (40)
L2	24 (38.7)	5 (16.7)
L3	36 (58.1)	13 (43.3)
L4	4 (6.5)	0
Behavior (montreal) (%)		
B1	34 (54.8)	15 (50)
B2	10 (16.1)	9 (30)
B3	18 (29)	7 (23.3)
Perianal disease	12 (19.4)	4 (13.3)
AZA (%)	40 (64.5)	22 (73.3)
6MERC (%)	6 (9.7)	0
Biologics (%)	20 (32.3)	2 (6.7)
Surgery (%)	25 (40.3)	13 (43.3)

*Complete clinical data was not available/reliable for 24 patients in Polish center; percentages are therefore calculated for 62 patients with completed clinical data

TABLE 2. *NOD2* genotype in patients with Crohn's disease and controls, n(%)

	NOD2 genotype						
	NOD2 Leu1007fsinsC (%)			*NOD2* Gly908Arg (%)		Any mutation (Leu1007fsinsC or Gly908Arg) (%)	Non-carrier (%)
	Heterozygous	Homozygous	Wild-type	GA	GG		
Poland							
CD (n=86)	15 (17.4)	13 (15.1)	58 (67.4)	2 (2.3)	84 (97.7)	29 (33.7)*	57 (66.3)
Controls (n=83)	5 (6.0)	0	78 (94.0)	4 (4.8)	79 (95.2)	9 (10.8)*	74 (89.2)
Bosnia							
CD (n=30)	0	3 (10)	27 (90)	0	30 (100)	3 (10)	27 (90)
Controls (n=30)	3 (10)	0	27 (90)	0	30 (100)	3 (10)	27 (90)
Total							
CD (n=116)	15 (12.9)	16 (13.8)	85 (73.3)	2 (1.7)	114 (98.3)	32 (27.6)	84 (72.4)
Controls (n=113)	8 (7.1)	0	105 (92.9)	4 (3.5)	109 (96.5)	12 (10.6)	101 (89.4)

*X^2=12.68; df=1; p<0.001

TABLE 3. Clinical characteristics of CD patients with respect to the presence of *NOD2/CARD15* mutations, n (%)

	Poland		Bosnia		Total	
	Any *NOD2* mutation (n=21)	Non-carrier (n=41)	Any *NOD2* mutation (n=3)	Non-carrier (n=27)	Any *NOD2* mutation (n=24)	Non-carrier (n=68)
Male/female	9/12	28/13	3/0	12/15	12/12	40/28
Age (yr)						
Age (montreal) (n;%)						
A1	2 (9.5)	5 (12.2)	0	1 (3.7)	2 (8.3)	6 (8.8)
A2	17 (81)	32 (78)	1 (33.3)	6 (22.2)	18 (75.0)	38 (55.9)
A3	2 (9.5)	4 (9.8)	2 (66.7)	20 (74.1)	4 (16.7)	24 (35.3)
Location (montreal) (n;%)						
L1	1 (4.8)	1 (2.4)	1 (33.3)	11 (40.7)	2 (8.3)	12 (17.6)
L2	6 (28.6)	18 (43.9)	0	6 (22.2)	6 (25.0)	23 (33.8)
L3	14 (66.7)	22 (53.7)	2 (66.7)	11 (40.7)	16 (66.7)	33 (48.5)
L4	2 (9.5)	2 (4.9)	0	0	2 (8.3)	2 (8.3)
Behavior (montreal) (n;%)						
B1	11 (52.4)	23 (56.1)	1 (33.3)	14 (51.9)	12 (50)	37 (54.4)
B2	4 (19)	6 (14.6)	0	9 (33.3)	4 (16.7)	15 (22.1)
B3	6 (28.6)	12 (29.3)	2 (66.7)	5 (18.5)	8 (33.3)	17 (25.0)
Perianal disease	1 (4.8)*	11 (26.8)*	1 (33.3)	3 (11.1)	2 (8.3)	14 (20.6)
AZA (n;%)	12 (66.7)	20 (75.7)	3 (100)	19 (70.4)	47 (73.4)	15 (71.4)
6MERC (n;%)	0	6 (16.2)	0	0	6 (9.4)	0
Biologics (n;%)	5 (23.8)	15 (36.6)	0	2 (7.4)	17 (25.0)	5 (20.8)
Surgery (n;%)	12 (66.7)**	13 (43.3)**	2 (66.7)	11 (40.7)	24 (42.1)	14 (66.7)

*Fisher's test; p=0.046; **X^2=3.73; df=1; p=0.05

The prevalence of any ***NOD2*** mutations in Polish sample of participants was significantly higher in CD patients (33.7%) than in healthy controls (10.8%). Although the sample of participants from Bosnia and Herzegovina was indeed too small, it is interesting to note that although both CD patients and controls in Bosnian sample had the same ratio of ***NOD2*** mutations (10%), all patients in CD group were homozygotes while those in control group were heterozygotes. Homozygotes are at much higher risk for developing CD when compared with heterozygotes, as mentioned before [11]. Cukovic-Cavka et al, reported similar ratio of ***NOD2*** mutations in the analysis of 136 CD patients (27.9%) and 91 healthy controls (10.9%) in Croatia [22]. In a similar study from Serbia, at least one ***CARD15*** disease-associated allele was found in 35.11% patients with CD and in 14.77% of healthy controls [23]. In a Czech population, these figures are somewhat higher, with at least one ***NOD2*** mutation in 46% CD patients and 21% in control subjects [24]. Nevertheless they are still comparable with studies coming from Slavic countries, including our results, as well.

Concerning the association between ***NOD2*** mutations and phenotypic characteristics of the disease, the results of our study have certain limitations. Although we confirmed the increased likelihood for surgery in Polish patients, other previously reported associations such as disease onset at younger age, isolated ileal disease, complicated disease (B2 or B3 according to Montreal classification) were not found to be associated with NOD2 mutations. [9, 22, 23, 28-31]. Surprisingly, we have demonstrated that the presence of ***NOD2*** mutations was significantly less frequent in CD patients with perianal disease. In the recent meta-analysis which included 49 studies with 8893 subjects, the association between the presence of ***NOD2*** mutations and phenotype of the disease has been found to be week in terms of its prognostic power [32]. However, the presence of two mutations in ***NOD2*** (either homozygous or complex heterozygous) had a high degree of specificity for an aggressive disease phenotype. In the same meta-analysis, although ***NOD2*** did not predict perianal disease, the results of the studies included in this particular subgroup analysis were heterogeneous with around half of the included studies showing reduction and another half showing increase in relative risk for perianal disease. In the subgroup analysis, a significant heterogeneity was demonstrated, suggesting unreliable degree of association of phenotype-genotype related to the presence of ***NOD2*** mutations, and disease behavior.

Important limitations of our study are that both samples (Polish and Bosnian) are small and there is a significant possibility that important associations between genotype and phenotype were not detected. Yet, CD is relatively rare disease [20]. Furthermore, clinical data was not available for all patients in Polish sample allowing us only to make an estimate of the prevalence of ***NOD2*** mutations without investigating associations with clinical characteristics. We investigated only 2 out of 3 most frequently sequenced types of ***NOD2/CARD15*** gene and it is possible that there are patients and controls with ***Arg702Trp*** mutation in our sample.

CONCLUSION

This is the first report on the prevalence of *NOD2/CARD15* mutations from Poland and B&H which demonstrates that the risk for CD is increased in Polish patients with *NOD2* mutations, and especially in the presence of homozygous *NOD2* mutations in both Polish and B&H patients. In addition, data obtained in Polish sample show that the presence of variant *NOD2* alleles is associated with increased need for surgery and reduced occurrence of perianal disease. Further studies with larger samples in both regions are warranted.

DECLARATION OF INTERESTS

The authors declare no conflict of interest.

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