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World J Gastroenterol 2005;11(3):407-411
World Journal of Gastroenterology ISSN 1007-9327

• BRIEF REPORTS •

NOD2/CARD15 gene polymorphism in patients with inflammatory bowel disease: Is Hungary different?

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Received: 2004-05-25 **Accepted:** 2004-07-17

Abstract

AIM: To analyse the impact of NOD2/CARD15 mutations on the clinical course of Crohn´s disease patients from an eastern European country (Hungary).

METHODS: We investigated the prevalence of the three common NOD2/CARD15 mutations (Arg702Trp, Gly908Arg, 1007finsC) in 148 patients with Crohn's disease, 128 patients with ulcerative colitis and 208 controls recruited from the University of Szeged, Hungary. In patients with Crohn´s disease, the prevalence of NOD2/CARD15 mutations was correlated to the demographical and clinical parameters.

RESULTS: In total, 32.4% of Crohn's disease patients carried at least one mutant allele within NOD2/CARD15 compared to 13.2% of patients with ulcerative colitis (P = 0.0002) and to 11.5% of controls (P<0.0001). In Crohn's disease patients, the allele frequencies for Arg702Trp, Gly908Arg and 1007finsC were 7.1%, 3.0% and 10.8% respectively. Interestingly, only the 1007finsC mutation was associated with a distinct clinical phenotype. The patients positive for the 1007finsC mutation suffered more frequently from stenotic disease (P = 0.008). Furthermore, 51.9% of patients positive for the 1007finsC mutation underwent a surgical resection within the ileum compared to only 17. 4% of patients without the 1007finsC mutation (P = 0. 001). With respect to the other two mutations (Arg702Trp and Gly908Arg), no associations were found with all investigated clinical parameters.

CONCLUSION: NOD2/CARD15 mutations are frequently found in Crohn's disease patients from Hungary. The 1007finsC mutation is associated with stenotic disease behaviour and frequent ileal resections.

Key words: Crohn's disease; NOD2/CARD15 gene; Mutation

Büning C, Molnar T, Nagy F, Lonovics J, Weltrich R, Bochow B, Genschel J, Schmidt H, Lochs H. NOD2/CARD15 gene polymorphism in patients with inflammatory bowel disease: Is Hungary different? *World J Gastroenterol* 2005; 11(3): 407-411
http://www.wjgnet.com/1007-9327/11/407.asp

INTRODUCTION

There is a strong support for the genetic influence on the pathophysiology of inflammatory bowel diseases (Crohn's disease and ulcerative colitis). Recent studies reported an association between Crohn's disease and mutations in the NOD2/CARD15 gene located on chromosome 16q12 (IBD1)[1-3]. NOD2/CARD15 acts as an intracellular receptor in monocytes for bacterial components triggering activation of NFκB thus leading to subsequent activation of the inflammatory response. These findings fit perfectly in the current working hypothesis that a dysregulated immune response towards antigens of fecal bacteria in a genetically susceptible host is the main pathogenetic feature in Crohn's disease.

Within the NOD2/CARD15 gene, three mutations have been identified to be associated with Crohn's disease: two missense mutations (Arg702Trp in exon 4 and Gly908Arg in exon 8) and an insertion mutation of a C in exon 11 (1007finsC), the latter resulting in a truncated NOD2/CARD15 protein. Numerous studies have been performed mostly in northern America and western Europe to determine the frequency of NOD2/CARD15 mutations in inflammatory bowel disease populations. In Crohn's disease patients from western Europe or northern America, allele frequencies ranged from 9.1-12.9%, 3.3-6.0% and 6.6-16.0% for Arg702Trp, Gly908Arg, 1007finsC, respectively[4]. Interestingly, a recent study in Finnish IBD patients reported lower allele frequencies for all of the three NOD2/CARD15 mutations in Crohn's disease patients compared the above-mentioned studies and furthermore similar frequencies in patients with ulcerative colitis[5]. Additionally, in Asian IBD populations NOD2/CARD15 mutations could not be detected at all[6,7]. This raises the need for more studies in different IBD populations. Up to now, no data is available on the occurrence of NOD2/CARD15 mutations in countries located between western Europe and Asia, e.g., eastern Europe. The first aim of our study was therefore to analyse for the first time the frequency of NOD2/CARD15 mutations in an eastern European country (Hungary). Patients with inflammatory bowel disease were recruited from the University of Szeged. Szeged is one of the largest cities in Hungary with a population of approximately 186 000. Located along the Tisza river and close to the border of Romania, Szeged is the cultural and economic center of South-Eastern Hungary.

With respect to the occurrence of inflammatory bowel disease in Hungary, a recent report from Lakatos and colleagues reported an increase of both Crohn's disease and ulcerative colitis in a province of Western Hungary from 1977 to 2001. In ulcerative colitis, the incidence increased from 1.66 to 11.01 and from 0.41 to 4.68 in Crohn's disease per 100.00 persons. Prevalence rates were reported to be 142.6 and 52.9 per 100.00 persons for ulcerative coltis and Crohn's disease respectively[8]. The incidence and prevalence rates of inflammatory bowel disease in Hungary are comparable to western European countries[9].

Interestingly, observations in Hungary suggest differences in disease prevalence among the Roma (Gipsy) minoritiy compared to the Caucasian population. Concentrated in some

Hungarian regions, the incidence of IBD within the Roma minority is half compared to the rest of the population living in these areas[10].

Crohn's disease shares a number of various phenotypes with differences in age at onset of disease, disease location and behaviour[11]. With respect to NOD2/CARD15 mutations, investigations have been performed to analyse the clinical impact of these mutations on Crohn's disease. Associations with ileal involvement[12-15], younger age at diagnosis[16], and fibrostenotic disease behaviour[17,18] have been described. However, only some of these associations were replicated by other investigators. In our recent observations in a large German cohort, we found an association with ileocecal resections and neoterminal reoperations[19]. The second aim of our study was, therefore to observe the genotype-phenotype correlation to answer the question whether a certain phenotype was associated with NOD2/CARD15 mutations.

In this study, we investigated the frequency of the three common NOD2/CARD15 mutations in 276 patients with inflammatory bowel disease and in 208 controls from Hungary. Furthermore, a detailed phenotype genotype analysis was performed to elucidate the clinical impact of NOD2/CARD15 mutations on the course of Crohn's disease.

MATERIALS AND METHODS

Patients

We included 148 patients with Crohn's disease in the study, who were diagnosed according to clinical, endoscopic, radiological and histological findings[20]. All patients were recruited from the 1st Department of Medicine, Faculty of Medicine, University of Szeged, Hungary. The study was approved by the Ethical Committee of the department. Informed consent was obtained from each participant.

Among the 148 patients, 138 were Caucasians and 10 were from the Roma minority (Gypsy). Clinical data were obtained from the patients clinical charts at the 1st Department of Medicine, University of Szeged, Hungary. Genotyping, statistical analysis and drafting of the manuscript were performed at the Charité, Berlin.

The following data of patients with Crohn's disease was collected: age, age at diagnosis, gender, familial or spontaneous disease (familial disease was considered if one first- or second-degree relative had IBD), smoking habits (current smoking/ history of smoking/never smoked), disease localisation, disease behaviour, perianal disease, extraintestinal manifestations (arthritis, affections of eyes or skin, primary sclerosing cholangitis), type and location of surgery. Disease localisation was defined as the maximum extent of digestive tract involvement at the latest followup. Information was obtained through endoscopic (including upper endoscopy and colonoscopy with multiple biopsies and histological examination) and radiological (small bowel x-ray or CT enteroclysma) examinations. Stenotic Crohn's disease was considered if persistent intestinal obstruction was found either in the small bowel x-ray, small bowel computer tomography or colonoscopy. Perforating disease was recorded if patients had enterocutaneous, enteroenteric, enterovesical or enterovaginal fistula, intraabdominal abscess or small bowel perforation. Inflammatory disease behaviour was considered if neither stenotic nor perforating disease behaviour was present. Perianal disease was considered if perianal fistulae, ulcers or abscesses were present.

The following extraintestinal manifestations were determinated: arthritis, presence of primary sclerosing cholangitis (PSC) diagnosed through endoscopic cholangiography, affections of skin (e.g., presence of erythema nodosum or pyoderma gangrenosum) or eye (e.g., presence of episcleritis or anterior uveitis).

Genotyping for the three NOD2/CARD15 mutations was also performed in 128 patients with ulcerative colitis (m:f 57:71; age 16-79 years). These patients were also recruited from the First Department of Medicine, University of Szeged, Hungary. A total of 208 unrelated Hungarian healthy individuals served as controls (m:f 96:112; age 18-86 years).

Polymerase chain reaction and sequencing

Genomic DNA from index patients and controls was prepared using commercially available extraction columns (QIAmp Blood Kit, QIAGEN, Hilden, Germany).

Exon 11 of the NOD2 gene was amplified using the following primers: forward primer 5'-ggg aca ggt ggg ctt cag ta-3', reverse primer 5'-cca ttc ctc tct ccc gtc ac-3'. The annealing temperature was 62 ℃. DNA sequencing of the amplified exon was performed by cycle sequencing with fluorescent dye terminators. Analysis was performed using an ABI 310 automatic sequencer (Applied Biosystems, Weiterstadt, Germany). The analysis was confirmed by sequencing in both directions.

SNP genotyping

Genotyping of the three most common NOD2/CARD15 SNPs (Arg702Trp, Gly908Arg, 1007finsC) was performed using the fluorogenic 5'-nuclease assay with the primers and probes listed in Table 1.

Each assay was performed using 25 μL IQ Supermix (BioRad, München, Germany), 0.6 μL of each primer (100 μmol/L), 0.1 μL of each probe (100 μmol/L) (TibMolBiol, Berlin, Germany), 3 μL genomic DNA, and 20.6 μL H_2O. Fluorescence was measured over 40 cycles, and allelic discrimination was performed with the ICycler IQ real-time PCR detection system and the appropriate software (BioRad, München, Germany).

Statistical analysis

Comparison of the frequency of NOD2/CARD15 mutations was made between patients and controls, and the analysis of association with the phenotype was performed by χ^2 test or Fisher's exact test when appropriate. For analysis of age and age at disease diagnosis, Wilcoxon-U-Mann-Whitney test was applied. *P* values less than 0.05 were considered statistically significant. The data was analysed using SPSS/PC+V10.01 software (SPSS, Chicago, USA).

Table 1 Primers and Probes used in genotyping of NOD2/CARD15 genetic variants

SNP	Primers	Probes	Annealing
Arg702Trp	TTCCTGGCAGGGCTGTTGTC	FAM-CCTGCTCCGGCGCCAGGC-TAMRA	64.5 ℃
	GTGGAAGTGCTTGCGGAGG	TET-CCTGCTCTGGCGCCAGGCC-TAMRA	
Gly908Arg	ACTCACTGACACTGTCTGTTGACTCT	FAM-TTTTCAGATTCTGGGGCAACAGAGTGGGT-TAMRA	
	AGCCACCTCAAGCTCTGGTG	TET-TTCAGATTCTGGCGCAACAGAGTGGGT-TAMRA	
3020insC	GTCCAATAACTGCATCACCTACCTAG	FAM-CCCTCCTGCAGGCCCTTGAAAT-TAMRA	62.5 ℃
	CTTACCAGACTTCCAGGATGGTGT	TET-CCTCCTGCAGGCCCCTTGAAA-TAMRA	

RESULTS

Distribution of NOD2/CARD15 mutations in Hungarian IBD patients

First, we investigated the frequency of the three NOD2/CARD15 mutations in 276 patients with inflammatory bowel disease and compared them to 208 controls. Genotypes and allele frequencies are shown in Table 2. A total of 32.4% of Crohn's disease patients carried at least one mutant allele within NOD2/CARD15 compared to 13.2% of patients with ulcerative colitis ($P = 0.0002$) and to 11.5% of controls ($P<0.0001$). The difference between patients with ulcerative colitis and controls was not statistically significant. With respect to the 1007finsC mutation, 18.2% of Crohn's disease patients were either heterozygous or homozygous compared to 4.7% of patients with ulcerative colitis ($P = 0.001$) and 4.3% of controls ($P<0.0001$). Although the other two mutations (Arg702Trp and Gly908Arg) were found in clearly higher frequencies in patients with Crohn's disease compared to patients with ulcerative colitis and controls, not all comparisons reached statistical significance (Table 2). Among the 10 Roma (Gipsy) patients, only one patient carried a mutation within NOD2/CARD15 (1007finsC).

Genotype phenotype analysis

In the next step a detailed genotype phenotype analysis was performed in the 148 patients with Crohn's disease. The percentages of Crohn's disease patients positive or negative for each individual NOD2/CARD15 mutation were correlated to demographic data, disease localisation, disease behaviour, extraintestinal manifestations and surgical interventions (Table 3).

The 1007finsC mutation was negatively correlated to inflammatory disease behaviour ($P = 0.001$) and positively associated with stenotic disease behaviour ($P = 0.008$). The frequency of ileal involvement was higher in patients positive for the 1007finsC mutation (66.7%) compared to patients negative for the 1007finsC mutation (51.2%), but this difference was not significant ($P = 0.20$).

We also analysed the correlation of frequency and location of surgical resections to the NOD2/CARD15 genotype. More than half of the patients positive for the 1007finsC mutation (51.9%) underwent a surgical resection of the ileum, while only 17.4% of patients without mutation were operated ($P = 0.001$). These findings could not be explained by different disease durations or disease locations in the two groups (Table 3). Furthermore, the association of the 1007finsC mutation with surgical interventions was specific for the ileal region since no differences were found when frequencies of large bowel resections were analysed (Table 3).

Table 3 Clinical characteristics of Crohn´s disease patients

Genotype	1007finsC (+)	1007finsC (-)
Total Number	27	121
Male: female	11:16	57:64
Age at diagnosis (yr)		
Range	13-46	12-65
mean±SD	25.9±8.0	30.1±12.8
Race, *n* (%)		
Caucasian	26 (96.3)	112 (92.6)
Roma (Gipsy)	1 (3.7)	9 (7.4)
Disease Duration (yr)	9.1±6.9	8.5±8.1
Familial Crohn´s disease, *n* (%)	1 (3.7)	9 (7.4)
Sporadic Crohn´s disease, *n* (%)	26 (96.3)	112 (92.6)
Smoking Habits, *n* (%)		
Never	15 (55.6)	69 (57.0)
Current	11 (40.7)	48 (39.7)
Ex-Smoker	1 (3.7)	4 (3.3)
Localisation, *n* (%)		
Upper GI	3 (11.1)	4 (3.3)
Ileum	18 (66.7)	62 (51.2)
Colon	20 (74.1)	103 (85.1)
Perianal Disease	7 (26.0)	5 (28.9)
Behaviour, *n* (%)		
Inflammatory	2 (7.4)[1]	47 (38.8)
Stricturing	17 (63.0)[2]	40 (33.0)
Penetrating	12 (44.4)	46 (38.0)
Surgery, *n* (%)		
Ileal resections	14 (51.9)[3]	21 (17.4)
Colonic resections	7 (26.0)	21 (17.4)
Fistulae	1 (3.7)	3 (2.5)
Extraintestinal manifestation, *n* (%)		
Arthritis	3 (11.1)	17 (14.0)
Primary sclerosing cholangitis	0	2 (1.7)
Skin	0	7 (5.8)
Eye	0	1 (0.8)

[1]Inflammatory disease behaviour was negatively correlated to the 1007finsC mutation ($P = 0.001$, Fisher's exact test). [2]Stenotic disease behaviour was significantly associated with the 1007finsC mutation ($P = 0.008$, Fisher's exact test). [3]Ileal resections were performed significantly more often in patients positive for the 1007finsC mutation ($P = 0.001$, Fisher's exact test). No other significant differences were found.

Table 2 NOD2/CARD15 genotypes and allele frequencies in Hungarian IBD patients

	Patients	Genotypes			Allele frequency (%)	*P* value[1]
		-/- *n* (%)	-/+ *n* (%)	+/+ *n* (%)		
Arg702Trp	CD	129 (87.2)	17 (11.5)	2 (1.4)	7.1	0.01[1]
	UC	120 (93.8)	8 (6.3)	0	3.1	0.07[2]
	Controls	197 (94.7)	11 (5.3)	0	2.6	n.s.[3]
Gly908Arg	CD	139 (93.9)	9 (6.1)	0	3.0	0.10[1]
	UC	124 (96.9)	4 (3.1)	0	1.6	0.27[2]
	Controls	203 (97.4)	5 (2.6)	0	1.2	n.s.[3]
1007finsC	CD	122 (82.4)	22 (14.9)	5 (3.4)	10.8	<0.0001[1]
	UC	122 (95.3)	6 (4.7)	0	2.3	0.001[2]
	Controls	199 (95.7)	9 (4.3)	0	2.2	n.s.[3]

Crohn's disease (CD, *n* = 148), ulcerative colitis (UC, *n* = 128) and controls (*n*=208); Genotypes: -/- homozygous wild-type, -/+ heterozygous mutant, +/+ homozygous mutant; Absolute numbers (percentages) of individuals negative (-/-), heterozygous (-/+), homozygous (+/+) for the different NOD2/CARD15 mutations (Arg702Trp, Gly908Arg and 1007finsC) are shown. Fisher's exact test was performed comparing percentages of patients positive or negative for the corresponding NOD2/CARD15 mutation between [1]CD and controls, [2]CD and UC, [3]UC and controls.

With respect to all other clinical and demographic data, including age at onset of the disease, disease duration, familial or sporadic disease, smoking behaviours or extraintestinal manifestations, no significant differences were found with respect to the 1007finsC genotype within NOD2/CARD15 (Table 3).

Additionally, genotype phenotype investigations performed with respect to the other two NOD2/CARD15 mutations (Arg702Trp and Gly908Arg) did not reveal any significant findings (data not shown).

DISCUSSION

This is the first study to investigate the frequency of NOD2/CARD15 mutations in an eastern European IBD population (Hungary). A total of 32.4% of Crohn´s disease patiens carried at least one mutant allele of the three investigated NOD2/CARD15 mutations. This is in agreement with the reported frequency of NOD2/CARD15 mutations in western European Crohn's disease populations[4]. In contrast, lower frequencies were reported in finnish IBD patients[5] and NOD2/CARD15 mutations have not been found at all in Asian patients[6,7].

We observed a significant association between the 1007finsC mutation and stenotic disease behaviour. Similar findings have been reported by other investigators[5,16-18]. In our study, this stenotic disease behaviour seemed to lead to more frequent surgical resections at ileal site, since approximately 52% of patients positive for the 1007finsC mutation had ileal surgery compared to about 17% of patients negative for the 1007finsC mutation. The association of ileal resections with mutations in the NOD2/CARD15 gene has been reported by other studies[17] and by our own observation in a large German cohort[19].

In contrast to the frameshift mutation 1007finsC, we were not able to detect any association with all clinical parameters tested for the other two common missense mutations Arg702Trp and Gly908Arg. Most of the studies performed so far have not discriminated between the individual impact of the three NOD2/CARD15 mutations on the clinical phenotype. Since the frameshift mutation could lead to a truncated NOD2/CARD15 protein, it is likely to be functionally more important than the other two mutations. This hypothesis is supported by recent functional studies by Bonen *et al*[21], who demonstrated that this frameshift mutation had a complete defect in response to bacterial components such as peptidoglycan (PGN), whereas Arg702Trp and Gly908Arg showed indermediate responses.

Furthermore, the 1007finsC mutation was negatively correlated to the inflammatory subtype (if neither strictures nor fistulae were present) of Crohn's disease. This has also been observed in another German Crohn's disease population[17]. There was a trend towards an association of the 1007finsC mutation with ileal involvement (1007finsC positive: 66.7%, 1007finsC negative: 51.2%), but it did not reach any statistical significance. Mutation within the NOD2/CARD15 gene and its association with ileal involvement have been reported by other investigators[12,14,15]. Apart from ileal involvement, investigators have also described a younger age at onset of the disease in patients homozygous or compound heterozygous for NOD2/CARD15 mutations[16], whereas our study and others failed to notice this phenomenon[13,15].

In our analysis, we observed higher frequencies of all the three investigated NOD2/CARD15 mutations in Crohn's disease patients compared to controls, which was in agreement with previous findings[4]. With respect to the Gly908Arg mutation, this difference did not reach any statistical significance ($P = 0.10$). The lack of significance has also been reported by other studies[22,23], but that could also be due to the low allele frequency and a smaller sample size in our study. Among the 10 patients from the Roma (Gipsy) minority, we only detected one NOD2/CARD15 mutation (1007finsC). As mentioned above, a lower incidence of IBD in the Roma population from Hungary was described[10]. Lower health consciousness, lower hygienic levels or a different genetic background among the Roma minority are some of the factors that could explain this phenomenon. Interestingly, the so called Musician Gypsies (because they live a westernised life) appear to have a similar incidence of inflammatory bowel disease as the general population[10]. However, since the sample size of Roma patients in our study was small, it could not be determinated whether NOD2/CARD15 mutations or any other genetic alteration contributed to the lower incidence of Crohn's disease in the Roma (Gipsy) minority.

In summary, NOD2/CARD15 mutations can be found in similar frequencies in Crohn's disease patients from Hungary compared to western European populations in whom only the 1007finsC mutation within NOD2/CARD15 can predict an aggressive phenotype with stenotic disease behaviour and frequent ileal resections.

ACKNOWLEDGEMENTS

We thank all patients who were involved in this evaluation.

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Edited by Wang XL