Original Paper

The Association between Nod2 R702w Polymorphism and Susceptibility to Colorectal Cancer in Romanian Patients

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ABSTRACT: It is well recognized that the inflammatory bowel disease (IBD) is associated with an increased risk of colorectal cancer (CRC). More susceptibility IBD genes have been reported, NOD2 being one of the most extensively investigated. The aim of this study was to evaluate a possible correlation between NOD2 rs2066844 C>T (also known as Arg702Trp or R702W) variant and CRC risk in a Romanian population. A total of 373 Romanian subjects (108 patients diagnosed with sporadic CRC and 265 controls) were enrolled in this hospital-based case-control study. The NOD2 R702W variants were detected by Real-time PCR using a predesigned TaqMan Genotyping Assay. The association between the genetic risk variant and CRC was expressed as odds ratios (OR) with 95% confidence intervals (CI). We did not find any statistically significant difference when we compared CC genotype with CT genotype (OR 1.1, 95% CI: 0.46-2.61; p=0.83) between CRC patients and controls. No TT homozygous genotype was detected. Also, we compared allele frequencies and no correlation was found (OR 1.09, 95% CI: 0.47-2.56; p=0.84). No association was found in the stratified analysis by tumor site, Dukes' stage and histological subtype. Our study suggests that the NOD2 R702W variant is not associated with CRC risk in the Romanian population. Further data from different and larger populations is required to determine whether NOD R702W SNP has effects on susceptibility to CRC.

KEYWORDS: *colorectal cancer, NOD2 receptor, gene polymorphism, genotype, susceptibility*

Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed cancer worldwide, ranking third in males and second in females, with a 10-fold variation in incidence across the world [1,2]. Although decreases in mortality rates were seen in many regions, CRC remains a major cause of cancer-related death in both sexes, with one of the highest rates in Central and Eastern Europe. These marked differences in incidence and mortality rates are related to the complex and mutifactorial etiology of CRC, involving host genetic susceptibility, enviromental factors (e.g. diet, obesity, smoking), inflammation and immune responses, and recently recognized microbiota [3,4].

Two major types of inflammatory bowel disease (IBD), Crohn's disease and ulcerative colitis are associated with an increased risk of CRC [5]. More susceptibility IBD genes have been reported, one of the most extensively investigated being NOD2 (nucleotide oligomerization domain 2), also known as CARD15 (caspase activating recruitment domain 15) [6-8]. NOD2 gene is located on the chromosome 16q12 within the IBD1 region and encodes a member of the cytosolic NOD like receptors (NLRs). NOD2 is an intracellular pattern recognition receptor that senses bacterial peptidoglycan by recognition of the muramyl dipeptide present in the bacterial cell wall [9,10]. NOD2 plays important roles in the regulation of gut microbiota in normal conditions and stimulates both innate and adaptive immune host responses against infectious pathogens, including bacteria, viruses and parasites [11].

Three major risk variants have been described in NOD2 gene, two missense mutations (Arg702Trp in exon 4 and Gly908Arg in exon 8) and one frameshift insertion mutation (3020insC of a C in exon 11) [6-8]. After the first report by Kurzawski et al. showing an association between 3020insC variant and the risk of CRC, more published studies in different ethnic populations have produced controversial results regarding the involvement of NOD2 genetic variations and CRC [12].

In this respect, the aim of this study was to investigate a possible correlation between NOD2 rs2066844 C>T (also known as Arg702Trp or R702W) variant and CRC risk in a Romanian population, an ethnic group from Eastern Europe in which the association between this polymorphism (SNP) and CRC susceptibility has not previously been studied.

Material and Methods

Subjects

A total of 373 Romanian subjects (108 patients diagnosed with sporadic CRC and 265 controls) were enrolled in this hospital-based case-control study. The CRC diagnosis was established by standard procedures and histopathological specimen examination, as we previously described [13]. All CRC patients were recruited from the Emergency Clinical Hospital County of Craiova, Romania. A control group of 265 individuals matched for ethnicity, sex, and age were randomly selected from unrelated volunteers admitted to the same hospital. All control subjects were without malignancy, inflammatory, autoimmune or infectious chronic disease. The Ethics Committee of University of Medicine and Pharmacy of Craiova, Romania approved this study and written informed consent was obtained from all the subjects.

DNA extraction and genotyping

Genomic DNA was extracted from the peripheral blood of all enrolled individuals using Wizard® Genomic DNA Purification Kit (Promega, Madison, WI), following the manufacturer's instructions. The NOD2 rs2066844 C>T SNP was detected using a predesigned TaqMan SNP Genotyping Assay (assay C__11717468_20, Applied Biosystems, Foster City, CA, USA) that includes two allele-specific TaqMan probes containing different fluorescent dyes and a PCR primer pair to target the region flanking the SNP site.

Reactions were completed and read using a ViiA™ 7 Real Time PCR System (Life Technologies, Carlsbad, USA). Thermal cycling conditions consisted of 1 cycle for 10 minutes at 95°C, 50 cycles for 15 seconds at 95°C, and 45 cycles for 1 minute at 60°C. To control the quality of genotyping, the reactions were performed without knowing the status of the cases or controls. The DNA samples were randomly distributed and each PCR run included a wild-type, a heterozygous, a mutant DNA control, and two negative controls.

Statistical analysis

Hardy-Weinberg equilibrium in both groups was analyzed by χ^2 test. The descriptive analysis of rs2066844 genotypes and clinicopathological features were expressed both in absolute values and percentages. Odds ratios (OR) with the corresponding χ^2 distribution test and 95% confidence intervals (95% CI) were used to assess the association between the investigated NOD2 variant and susceptibility to CRC. The relationship between the NOD2 R702W variant and CRC risk was analyzed under heterozygote (CT vs. CC) and allelic (T vs. C) models. Homozygous genotype for the wild-type allele in Caucasians was used as the reference category. Only *P* values <0.05 were considered significant. All data were analyzed by SPSS software version 17.

Results

We successfully genotyped NOD2 R702W in 108 patients diagnosed with CRC and 265 controls. The characteristics of both groups are summarized in Table 1. Cases and controls were well matched, and no significant differences in sex and age were observed between the patients and controls (p>0.05). CRC cases were characterized by tumor site, Dukes' stage and histologic grade. Based on CRC subsite location, 73 cases were colon and 35 rectal cancers, respectively. The tumor confined to mucosa and submucosa (A) in 3 cases, in 51 cases the tumor has penetrated the muscle wall of the bowel (B), in 38 cases the tumor had invaded regional lymph nodes (C), and distant metastasis were observed in 16 cases (D). According to the cancer cell differentiation, a number of 27 CRC cases were well differentiated, 58 were moderately differentiated and 23 were poorly differentiated.

The observed NOD2 R702W genotype frequencies in cases and controls were in agreement with Hardy-Weinberg equilibrium (cases: χ^2=0.16, p=0.69; controls: χ^2=0.32, p=0.57), suggesting no population stratification.

NOD2 R702W risk variant (T allele) was found in 8 CRC patients (7.41%) and 18 controls (6.79%) (Table 2). No homozygous were identified, all R702W carriers were heterozygous. We did not find a statistically significant difference between CRC patients and controls when we compared CC genotype with CT genotype (OR 1.1, 95% CI: 0.46-2.61; p=0.83). Furthermore, minor T allele frequencies (3.7% vs. 3.4%) were not statistically different between patients and controls (OR 1.09, 95% CI: 0.47-2.56; p=0.84). The association of R702W with tumor site, Dukes' stage and histological subtype was examined separately. No correlations were observed between tumor site (colon, rectum), tumor stage (A+B, C+D) or histological grading and controls in the stratified analysis (Table 3).

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Table 1. Patients characteristics

	Colorectal cancer	Control
N	108	265
Male/Female	65/43	143/90
Age (years), mean±SD	66,5±7,71	63,69±7,94
Location		
- cecum	8	
- sigmoid	35	
- descending	9	
- transverse	7	
- ascending	14	
- rectum	35	
Tumor stage-Dukes stage		
- A + B	3+51	
- C + D	38+16	
Differentiation Grade		
- G1-well	27	
- G2-moderate	58	
- G3-poor	23	

Table 2. NOD2 R702W frequencies and the association with susceptibility to colorectal cancer

	Colorectal cancer (n=108)	Control (n=265)	OR (95% CI)	p
NOD2 R702W				
CC	100 (92.59%)	247 (93.21%)	Reference	
CT	8 (7.41%)	18 (6.79%)	1.1 (0.46-2.61)	0.83
TT	0 (0 %)	0 (0 %)	-	
C:T	96.30%: 3.70%	96.06%: 3.40%	1.09 (0.47-2.56)	0.84

Table 3. Comparative analysis between NOD2 R702W genotype frequencies and the risk of colorectal cancer in the stratified analysis

	NOD2 R702W			
	CC	CT	TT	OR (95%CI); p
Tumor site				
- Colon (73)	67 (91.78%)	6 (8.22%)	0 (0%)	1.23 (0.47-3.22); 0.67
- Rectum (35)	33 (94.29%)	2 (5.71%)	0 (0%)	0.83 (0.19-3.75); 0.81
Histological grade				
-G1 (27)	24 (88.89%)	3 (11.11%)	0 (0%)	1.71 (0.47-6.24); 0.41
- G2 (58)	55 (94.83%)	3 (5.17%)	0 (0%)	0.75 (0.21-2.63); 0.65
- G3 (23)	21 (91.30%)	2 (8.70%)	0 (0%)	1.31 (0.28-6.01); 0.73
Tumor stage-Dukes				
A+B (54)	49 (90.70%)	5 (6.79%)	0 (0%)	1.40 (0.49-3.95); 0.52
C+D (54)	51 (94.44%)	3 (5.56%)	0 (0%)	0.81 (0.23-2.84); 0.74

Discussion

In this study, we evaluated whether the NOD2 R702W variant has an effect on susceptibility to CRC in a Romanian population. We did not find any significant association between this SNP and overall CRC risk or in the stratified analysis by tumor site, tumor stage and histological grading.

Published findings among different ethnic groups are controversial, some studies found a positive association whereas others were not able to reproduce it. Our results are similar to other research conducted in different racial groups. In this line of work, no correlation was found between R702W variant and susceptibility to CRC in Hungarian [14,15], respectively. NOD2 SNPs including R702W were not associated with CRC susceptibility in the

Malaysian patients [16]. In addition, NOD2 R702W not influenced susceptibility to CRC in a German cohort, but for the subgroup patients aged under 50 with early disease manifestation a significant association was found [17].

In contrast, NOD2 R702W variant was associated with a 5-fold higher risk for CRC in a Greek cohort, suggesting that this SNP can be a predisposing factor to sporadic CRC [18]. Also, a significant increased risk for CRC was observed for carriers of R702W in a Portuguese population, mainly for patients diagnosed under 60 years old and women [19]. The frequency of R702W was significantly higher in CRC patients compared with controls in a New Zealand population [20]. Moreover, this SNP was associated with a higher risk of CRC in two meta-analyses [21,22].

The frequency of alleles of NOD2 R702W varies considerably between races and geographic areas [23]. We have to note that the minor allele frequency for both CRC patients and controls in our study was higher than those found in Hungarian and Finnish populations and comparable to those previously reported in New Zealand, Portugal and Germany (Table 4).

Table 4. Comparation of minor allele frequencies of the NOD2 R702W SNP in different populations

Country	Allele frequency		Reference
	Cases (%)	Controls (%)	
Romania	3.7	3.4	Present study
Hungary	1.8	1.5	[14]
Finland	2.2	2.1	[15]
Germany	5.1	4.6	[17]
Greece	4.8	1	[18]
Portugal	6.7	2.6	[19]
New Zeeland	7.1.	3	[20]

Whereas our results suggest that R702W variant has no effect on CRC risk in Romanian patients, we cannot exclude a role for NOD2 as a low penetrance CRC susceptibility gene in other populations.

There might be also other modifying factors that can confer an increased NOD2 related risk of CRC in different ethnic groups, but not in the Romanian population.

Also, environmental factors and additional genetic factors vary greatly between populations.

Another explanation for our results could be the regional heterogeneity among individuals of different ethnic groups or geographical areas within European populations, similar to Crohn's disease susceptibility, reflecting the effects of differing founder populations [24].

The potential limitations of our study should be mentioned.

Firstly, the size of the study population is relatively small and biased selection of cases and controls may occur.

Secondly, we cannot rule out the fact that other genes involved in the immune or inflammatory responses may also contribute to the risk of disease.

Also, the potential effect of environmental factors and/or their interaction with NOD2 mutations have not been evaluated in the present study.

Conclusion

Our study suggests that the NOD2 R702W variant is not a predisposing factor to sporadic CRC in the Romanian population.

Further research is required in different and larger populations to determine whether NOD R702W SNP has effects on susceptibility to CRC.

Author contribution

*****Florin Burada and Cecil Sorin Mirea contributed equally to this study

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Conflict of interest

No conflict of interest to declare.

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