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Full Length Research Paper

CARD15/NOD2 polymorphisms differ among Polish Crohn's disease (CD) and differentiated thyroid cancer (DTC) patients

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Recent studies proved that mutations in the *CARD15/NOD2* gene can increase the risk for different malignancies, e.g. bowel, lung and breast cancer. Although many correlations between *CARD15/NOD2* and malignancies have been investigated, so far no association between inflammatory bowel diseases and differentiated thyroid cancer (DTC) among Polish patients have been done. The aim of this study was to investigate two polymorphisms in CARD15/NOD2 gene: rs2066842 (p.Pro268Ser, c.802C>T), rs2066845 (p.Gly908Arg, c.2722G>C) and one frameshift mutation rs5743293 (p.Leu1007fsinsC, c.3019_3020insC) among two groups of patients: Crohn's disease and differentiated thyroid cancer. A total of 591 individuals were enrolled in this study: 193 DTC patients (35 men and 158 women), 185 CD patients (96 women, 89 men) and 213 individuals in population group (107 male and 106 female). Genotyping and statistical analysis for all the investigated individuals was performed. This study confirmed the results obtained in previous investigations about the role of *CARD15/NOD2* gene in CD, but did not revealed relations between *CARD15/NOD2* gene and DTC.

Key Words: inflammatory bowel diseases, differentiated thyroid cancer, CARD15/NOD2

INTRODUCTION

Since Hugot et al. (1) defined in 1996 susceptibility loci for Crohn's disease in locus 16q12 of human genome (IBD1), research investigation started. It caused in 2001 finding *CARD15/NOD2* gene (2). Next years, many further investigations were done, which allowed e.g. to find out changes in *CARD15/NOD2* gene sequence. Research investigations resulted in finding two polymorphisms known as Pro268Ser (rs2066842), Gly908Arg (rs2066845) and one mutation - Leu1007fsinsC (rs5743293), which in later studies were expected to play a major role in the background of IBD. Carrying these changes may increase the risk for CD

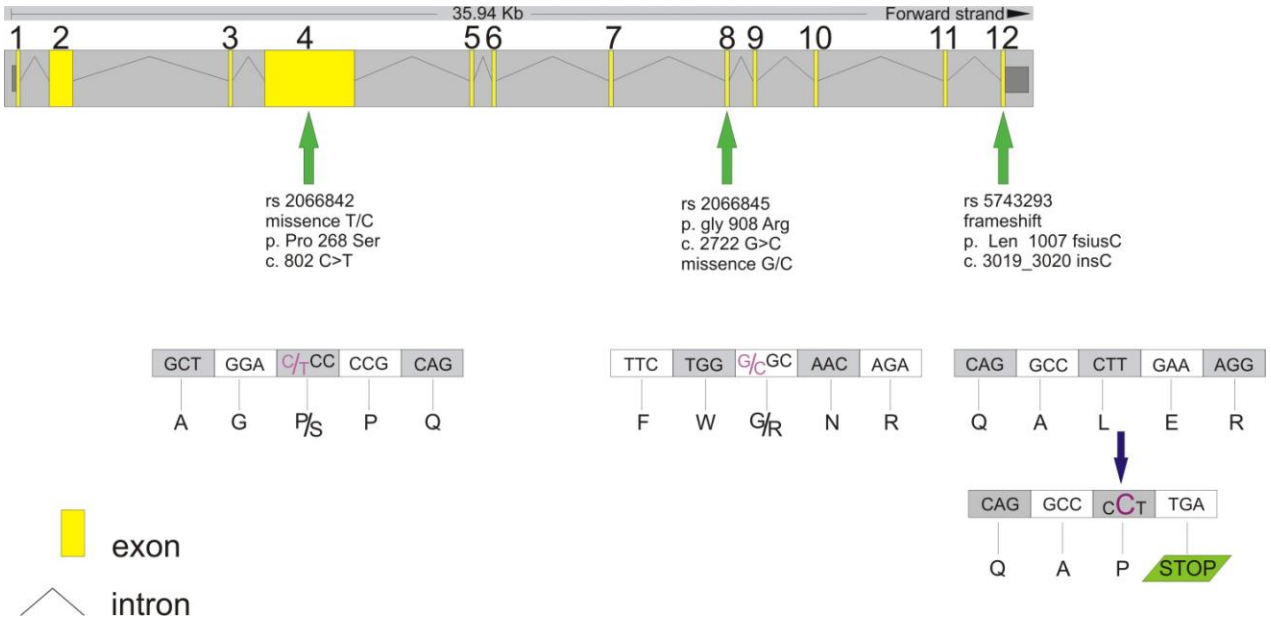


Figure 1. *CARD15/NOD2* gene. Analyzed mutation and polymorphism are marked. (Figure by L. Jakubowska-Burek).

even 20 times (carrying the 3020insC mutation increases the risk for malignancy from 3 (heterozygotes C/-) to even 20 times (for homozygotes C/C) (2). Ogura et al. proved also that 1007 frame-shift mutations in *CARD15/NOD2* gene are closely related to Crohn's disease, increases risk for cancerogenous changes among patients over 50 y.o.l., and contribute to ulcers (3). What is interesting, differences in frequency among different populations can be observed. For example, in Asian population R702W, G908R and 1007fs polymorphisms were not declared (4).

Recent studies proved also that mutations in the *CARD15/NOD2* gene can increase the risk for different malignancies, e.g. bowel, lung and breast cancer (5). Although many correlations between *CARD15/NOD2* and malignancies have been investigated, so far no association between inflammatory bowel diseases and differentiated thyroid cancer (DTC) among Polish patients have been done.

Although inflammatory bowel diseases (IBD) are different types of human disorders/diseases than differentiated thyroid cancer (DTC), it can also lead to development of malignancies. It is known, that CD is a disorder of gastrointestinal (GI) trackt caused both by genetic and environmental factors. Starting with bloody stools, diarrhea, pain, elevated CRP (C-reactive protein) and body temperature, turns into fistulas and ulcers - typical symptoms of IBD. With such serious symptoms risk for developing the colorectal cancer (CRC) is significantly increased, especially among patients touched by the disease for many years.

On the other hand, thyroid cancers are the most often malignancies of endocrine system (6). Thyroid cancers account for 1% of all malignancies in Polish population. Clinically, they can be divided into differentiated and undifferentiated. Differentiated thyroid cancers (DTC) are more frequent - constitute 80-90% of all patients – and are divided into follicular and papillary. Other type of thyroid cancer is medullary thyroid cancer, which can be both inherited and sporadic. As background of the medullary thyroid cancer is known and connected with mutations in *RET* protooncogene, so far background of DTC is still under investigation. DTC are rare among children and older people, but affect patients mostly between 25-65 year of life. What is more, thyroid cancers affect women three times more frequent than men (6,7) .

Although mutations and polymorphisms present in *CARD15/NOD2* gene were investigated according to different diseases in Polish population, e.g. colorectal cancer (8), primary sclerosing cholangitis, primary biliary cirrhosis (9), breast and lung cancer (10), so far changes in *CARD15/NOD2* gene in accordance to differentiated thyroid cancer were not considered yet.

Aim of the Study

The aim of this study was to investigate two polymorphisms in *CARD15/NOD2* gene: rs2066842 (p.Pro268Ser, c.802C>T), rs2066845 (p.Gly908Arg, c.2722G>C) and one frameshift mutation rs5743293 (p.Leu1007fsinsC, c.3019_3020insC) among two groups of patients: Crohn's disease and differentiated thyroid cancer (Figure 1). We want to find out if there are any correlations/associations between frequency of the genotypes and alleles, according to different groups of patients.

Table 1. Primers used for *CARD15/NOD2* analysis.

Primer	Sequence **5'□3'**	Product of PCR in bp	Annealing temp.°C
rs2066842F(p.Pro268Ser, c.802C>T) biotin	GTACCTATGATGGAGCAGAGACG		
rs2066842 R	AGCGTGCTCTTGCCACTG	211	64
rs2066842 seq	GGGCTCTTCTGCGGG		
rs2066845F(p.Gly908Arg,c.2722 G>C) biotin	GACTCTTTTGGCCTTTTCAGATT		
rs2066845 R	CCAATGGTCTTTTTTCCTTACTCC	243	58
rs2066845 seq	TCGTCACCCACTCTGT		
rs5743293F(p.Leu1007fsinsC,c.3019_3020insC)	ACCTACCTAGGGGCAGAAGC		
rs5743293 R biotin	CAGACTTCCAGGATGGTGTCA	66	58
rs5743293 seq	CCCTCCTGCAGGCCC		

MATERIALS AND METHODS

A total of 591 individuals were enrolled in this study: 193 DTC patients (35 men and 158 women), 185 CD patients (96 women, 89 men) and 213 individuals in population group (107 male and 106 female). All the patients came from Poznan University of Medical Sciences, Poland: DTC patients came from Department of Endocrinology, Metabolism and Internal Diseases, while CD patients came from Department of Gastroenterology, Human Nutrition and Internal Diseases. Population group belongs to bank of DNA in Institute of Human Genetics Polish Academy of Sciences. All persons examined in this research project were adult and agreed to take part in genetic testing.

Peripheral blood samples were collected on EDTA in the amount of 5 ml. Then, DNA was isolated from leukocytes using method with GTC (guanidine thiocyanate) and phenol-chlorophorm extraction. Isolates were dissolved in 1xTE buffer to obtain 500 ng/µl concentration.

For SNP (single nucleotide polymorphism) investigation pyrosequencing was performed. Analysis was performed for each probe with the set of primers: two for PCR (polymerase chain reaction), including one biothynylated, and one for sequencing. Sequences of the primers were designed by using PyroMark Assay Design Software (Table 1).

Statistical analysis was performed using the exact Fisher test and the test for differences between two frequencies. The odds ratio (OR) was also determined. A p-value of less than 0.05 was accepted as statistically significant.

RESULTS

Genotyping was performed for all individuals, but sometimes obtaining the certain result was impossible (probe contamination, unreliable result). In such cases results that were not sure were removed from further analysis. That is why sometimes the amount of obtained results differ from initial amount of investigated probes.

CARD15/NOD2 Pro268Ser

Analysis of *CARD15/NOD2* gene among patients suffering from Crohn's disease (CD) and differentiated thyroid cancer (DTC) revealed significant differences in genotype and alleles frequency (Table 1). The distribution of genotypes in *CARD15/NOD2* Pro268Ser polymorphism was non-random (p=0.0055). In CD group the most frequent genotype was heterozygous AG (47,8%) *(*in population group 57,1%). These frequencies were statistically significant (p=0.0133). Similarly, differences in GG genotype among CD and DTC patients were also statistically significant (p=0.0015). General trends of genotypes frequency in DTC patients group were different than for CD and population group (Figure 2) – the most frequent was GG genotype. Odds ratio (OR) was also determined (Table 2).

The odds ratio analysis revealed, that in patients with GG genotype a risk for developing the CD is 0.501 times as the risk for developing the DTC.

So if the odds ratio equals 4, the disease occurs four times as often in people exposed to the risk factor as in people not exposed.

Patients with AG genotype have 1.740 times bigger risk for developing the CD than DTC.

The analysis of alleles confirmed the results of the analysis of genotypes (Table 3); the most frequent for all groups was allele G (Figure 3), which was observed among DTC patients in 72,6% and among CD patients in 62,2%.

Odds ratio was also determined and revealed, that patients with allele G have 0.7978 times bigger risk for developing CD than DTC (OR=0.7978).

Analysis of insertion mutation in *CARD15/NOD2* gene revealed that among CD patients the insertion of cytosine was significantly more frequent than in population group. Monoalllelic/heterozygotic insertion in CD patients was observed among 16,8% of patients, while diallelic/homozygotic insertion of cytosine carried 4,9% of patients. What is interesting, 3020insC mutation was present in DTC patients also but only among 6,7% of patients, which was less than among general population (8,5%) (Table 4). What is more, among DTC group we

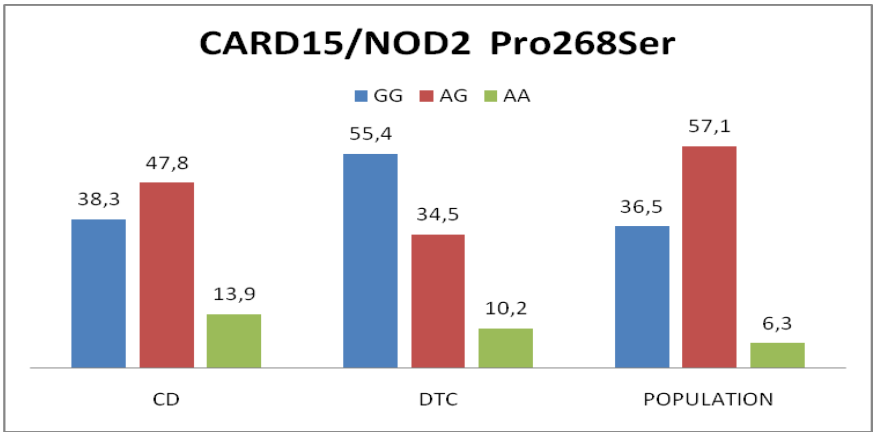


Figure 2. *CARD15/NOD2* Pro268Ser genotypes frequency

Table 2. *CARD15/NOD2* Pro268Ser genotypes analysis results

Pro268Ser	CD	%	DTC	%	POPUL.	%	P
GG	69	38,3	98	55,4	69	36,5	0.0015
AG	86	47,8	61	34,5	108	57,1	0.0133
AA	25	13,9	18	10,2	12	6,3	p>0.05 (ns)
Total	180	100,0	177	100,0	189	100,0	0.0055

Table 2. *CARD15/NOD2* Pro268Ser genotypes comparison.

Genotypes comparison	**OR**
GG vs AG or AA	0.501
AA vs AG or GG	1.425
AG vs AA or GG	1.740

Table 3. *CARD15/NOD2* Pro268Ser alleles analysis results

Pro268Ser	CD	%	DTC	%	POPUL.	%	p
G	224	62,2	257	72,6	246	65,1	p=0.0045
A	136	37,8	97	27,4	132	34,9	p=0.0017
Total	360	100,0	354	100,0	378	100,0	p=0.0003

did not observe any homozygotic insertion (C/C genotype) (Figure 4).

Odds ratio analysis revealed slightly increased risk for developing the disease according to adequate genotype (Table 5).

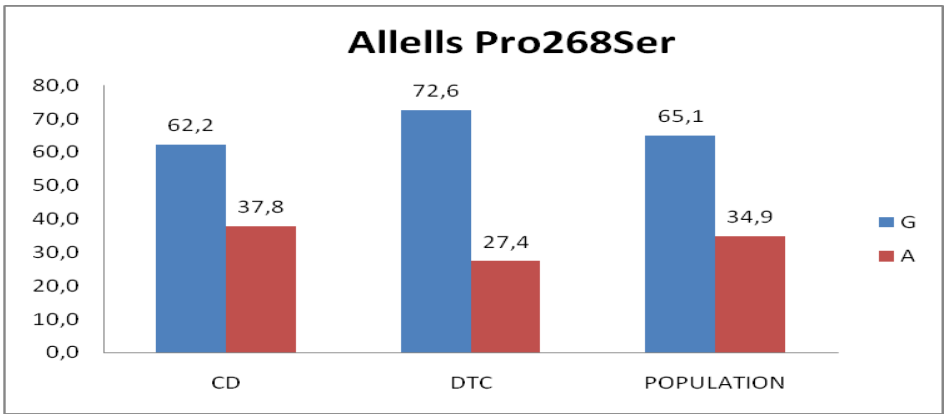
Odds ratio analysis revealed, that homozygotic insertion of cytosine (C/-) increases the risk for developing CD rather than DTC almost three times (OR=2.960), while when the patients have insertion in both alleles the risk is bigger than 23 times (OR=23.570).

Analysis of alleles in Leu3020insC mutation in CARD15/NOD2 gene revealed that the insertion of cytosine is observed in all investigated groups, although it is significantly more frequent among CD patients (13,2%) than DTC (3,4%) and population group (4,2%) (Table 4).

CARD15/NOD2 Gly908Arg

Analysis of *CARD15/NOD2* gene Gly908Arg polymorphism revealed significant differences in frequency of genotypes (Table 6). Genotype CG was observed more frequent in CD patients group (12,2%) than in population group (6%), while among DTC patients

Figure 3. Allele frequency for *CARD15/NOD2* gene Pro268Ser



CARD15/NOD2 Leu3020insC

Table 4. *CARD15/NOD2* Leu3020insC genotypes analysis results

Leu3020insC	CD	%	DTC	%	POPUL.	%	P
-/-	145	78,4	180	93,3	195	91,5	0.002
C/-	31	16,8	13	6,7	18	8,5	0.0008
C/C	9	4,9	0	0,0	0	0,0	Ns
Total	185	100,0	193	100,0	213	100,0	-

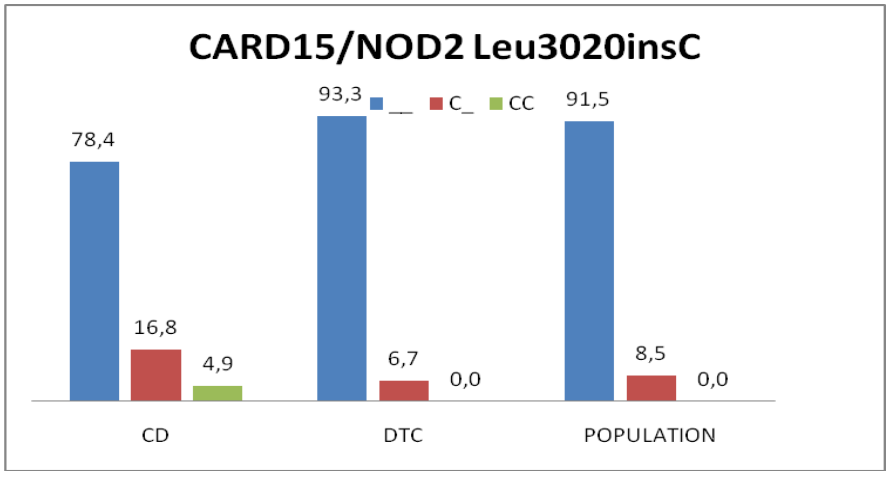


Figure 4. *CARD15/NOD2* Leu3020insC genotypes frequency

the percent of CG genotype was lower than in population group (4,2%). GG genotype was not observed at all among investigated individuals (Figure 5).

Alleles distribution presented the same tendency as genotypes frequency – allele G was the most often among CD patients (6,1%), while in DTC patients it was more rare than in population group (2,1% and 3%, respectively) (Figure 6), but the most often generally was allele C for both investigated groups with p=0.0094 and OR=0.3316 (Table 7).

Table 5. *CARD15/NOD2* 3020insC alleles analysis result

3020insC	CD	%	DTC	%	POPUL.	%
-	321	86,8	373	96,6	408	95,8
C	49	13,2	13	3,4	18	4,2
Total	370	100	386	100	426	100

Table 6. Statistical analysis for *CARD15/NOD2* Gly908Arg polymorphism among CD and DTC patients

Gly908Arg	CD	%	DTC	%	POPUL.	%	P	OR
CC	137	87,8	182	95,8	140	94,0	0.0020	0.3169
CG	19	12,2	8	4,2	9	6,0	0,0052	
GG	0	0,0	0	0,0	0	0,0	-	-
Total	156	100,0	190	100,0	149	100,0	-	-

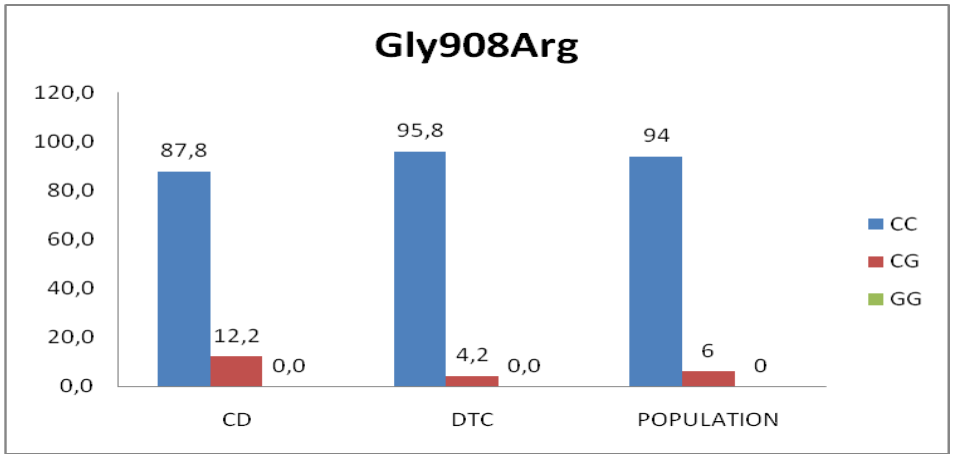


Figure 5. *CARD15/NOD2 Gly908Arg* genotypes frequency

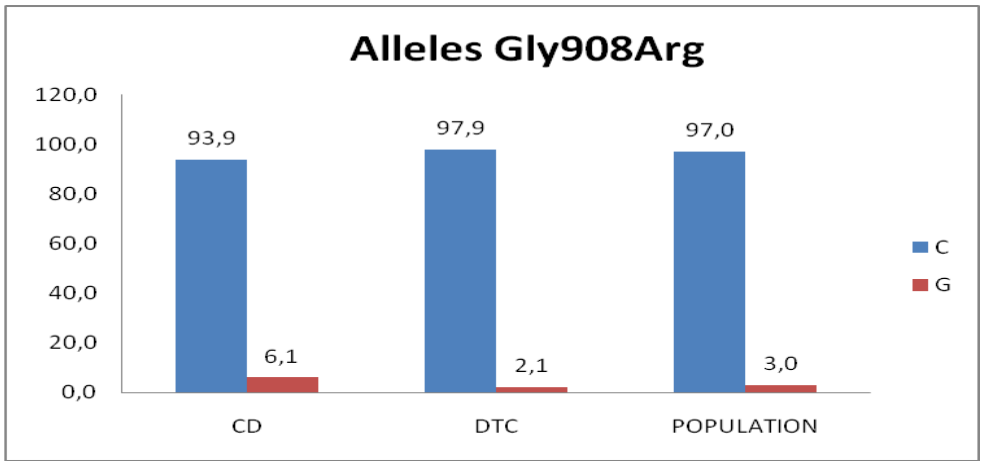


Figure 6. Alleles distribution in *CARD15/NOD2* Gly908Arg polymorphism

Table 7. *CARD15/NOD2* Gly908Arg alleles analysis results

Gly908Arg	CD	%	DTC	%	POPUL.	%	p	OR
C	293	93,9	372	97,9	289	97	p=0,0094	0.3316
G	19	6,1	8	2,1	9	3		
Total	312	100,0	380	100,0	298	100,0	-	-

DISSCUSSION

In many research projects that were run so far, the role of *CARD15/NOD2* protein was related with its significant role in reaction to bacterial lipopolisacharides. It's role in apoptosis (known also as programmed cell death), as in activating the NF-kB factor, responsible for transcription of many genes coding cytokines engaged in metabolic proinflammatory pathways (11) is also known. About ten years ago (2) Hugot published results where proved, that homozygotic pattern of 3020insC mutation (insertion of cytosine in one of the alleles) is connected with three-times increased risk for CD, while homozygotic insertion (insertion of cytosine in both alleles) increases the risk even 20 times. Insertion of cytosine in 3020 position of mRNA was also responsible for higher risk for malignancies, especially among patients above 50 year of life. Carriers of this mutation have also bigger risk for gangrenous lesions. In another study investigating prevalence of the NOD2 3020insC mutation in aggregations of breast and lung cancer in Polish population (8). Lener et al 2006 suggest also relation between the occurrence of 3020insC mutation and presenting the breast cancer among women who wave 1st or 2nd line relative with lung cancer (ca 15% of Polish patients). In a different study the 3020insC was expected to act as a low risk modifier of colorectal cancer (CRC) risk (8), although in different study the authors did not bound the presence of CRC with 3020insC mutation (12). Some correlations between *CARD15/NOD2* and malignancies in Polish population have been investigated, e.g. colorectal cancer (8), primary sclerosing cholangitis, primary biliary cirrhosis (9) and breast and lung cancer (10), so far changes in *CARD15/NOD2* gene in accordance to differentiated thyroid cancer were not considered.

This study investigates two groups of patients: CD and DTC, and compares the obtained results to population group. Although some significant differences in frequencies were observed, it cannot be said that the two polymorphisms Pro268Ser and Gly908Arg and one insertion mutation Leu3020insC have significant influence on DTC. This study confirmed previous investigations, which declared the role of 3020insC in the background of CD. Although we observed this mutation among DTC patients, it does not seem to play significant role in DTC. Having any of the three changes in DNA sequence (p.Pro268Ser, p.Gly908Arg, p.Leu1007fsinsC) increases more the risk for developing the CD than DTC. Alleles analysis confirmed the results obtained for genotypes analysis.

It is also worthy seeing, that although the spectacular results were not obtained, it is known how the frequency of p.Pro268Ser, p.Gly908Arg and p.Leu1007fsinsC looks like among DTC patients.

CONCLUSION

This study confirmed the results obtained in previous investigations about the role of *CARD15/NOD2* gene in CD, but did not revealed relations between *CARD15/NOD2* gene and DTC.

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