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ORIGINAL ARTICLE

NOD2 exonic variations in Iranian Crohn's disease patients

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Abstract
Purpose The *NOD2* gene is known to have a strong association with Crohn's disease, but different trends were reported in occurrence of *NOD2* variants in distinct ethnicities. The aim of this study was to assess all exonic sequences of the *NOD2* gene in Iranian Crohn's disease patients and healthy controls to identify any existing variation and evaluate their association with Crohn's disease.
*Methods*A total of 90 non-related Crohn's disease patients and 120 sex- and age-matched healthy controls of Iranian origin were enrolled in this study. The participants were referred to a tertiary center in a 2-year period (2006–2008). The exonic regions of the *NOD2* gene were amplified by polymerase chain reaction and evaluated by direct sequencing.
Results A total of 21 sequence variations were identified among all exonic regions of the *NOD2* gene, of which eight had an allele frequency of more than 5%. Eight new mutations (one in exon 2 and seven in exon 4) were observed. The three main variants (R702W, G908R, and 1007fs) showed allele frequencies of 13.3%, 2.2%, and 1.7%, respectively. Three new variations (P371T, A794P, and Q908H) and R702W mutation were significantly more frequent in Crohn's disease patients compared to controls.
Conclusions Eight novel mutations were identified in the *NOD2* exons, but the pathophysiological importance of these variants remains unclear. Iranian patients with their different genetic reservoirs may demonstrate some novel characteristics for disease susceptibility.

Keywords *NOD2* · Crohn's disease · Mutation · Sequencing

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Introduction

Crohn's disease (CD), a major form of inflammatory bowel disease (IBD), is a chronic granulomatous inflammation of the gut. It may involve any portion of the gastrointestinal (GI) tract and could affect all layers of the digestive wall [1]. The exact pathogenesis of CD is still unknown; but it is thought to result from inappropriate activation of the mucosal immune system in response to the antigens of intestinal bacteria [2].

The role of genetic factors in CD was first suggested by epidemiologic data and confirmed by familial aggregation and monozygote twin studies [3]. Microsatellite markers were then used to identify the areas of linkage with CD in the whole genome. In these challenges, several loci have been marked to be linked with CD [4, 5]. One of the strongest associations was observed with a locus on chromosome 16q12 assigned as IBD1 [4]. This locus contains the first reported CD linked gene, *NOD2* [6–8]. NOD2 protein, the product of this gene, is a cytosolic pattern recognition receptor responsible for regulation of innate immunity by intracellular recognition of the micro-organisms' particles such as lipopolysaccharides [9]. Moreover, NOD2 protein plays a role in apoptosis, nuclear factor

kappa B (NF-kB) activation, and development of inflammatory response in epithelial cells. As NF-kB has a key role in inflammation, NOD2 can be considered as a pro-inflammatory protein [10]. Structurally, it contains two caspase recruitment domains (CARDs), a nucleotide-binding oligomerization domain, and a C-terminal leucine-rich repeat (LRR) [11].

Many studies were performed to clarify the *NOD2* gene's role in IBD pathogenesis. Different variations of this gene were reported to be associated with CD in Caucasian populations [12–17]. Three common mutations of the *NOD2* gene (R702W, G908R, and 1007fsinsC) showed a significant association with CD in many European and American studies [18] while the lack of association was reported in other ethnicities such as Japanese and Koreans [19]. In a recent study, only one of the three common *NOD2* variations (R702W) was reported to be associated with CD in Iranian population, and two other variations demonstrated a very low frequency (<5%) in both patients and controls [20]. These results raised the idea that other variations of the *NOD2* gene might be responsible for susceptibility to CD among this ethnic group. Considering the rising number of affected individuals in Iran [21] and previously reported associations of other gene variants (such as *MDR1* and *VDR*) with CD, as well as different trends of the NOD2 common variations in this ethnicity [22, 23], we evaluated all exonic regions of the *NOD2* gene in Iranian CD patients and healthy controls to identify any existing variations and assess any probable association of them with CD.

Methods and materials

In this case–control designed study, a total of 90 unrelated CD patients as well as 120 age- and sex-matched healthy controls, all of Iranian origin, were recruited. Patients were referred to a tertiary GI center in Tehran (the capital of Iran) in a two-year period (2006–2008). CD was diagnosed based on the clinical, endoscopic, radiologic, and histopathologic findings [1, 24, 25] by an expert gastroenterologist. A complete clinical questionnaire was filled for each patient at admission. CD phenotype was categorized according to Vienna classification including disease location, behavior, and age at diagnosis [26]. Controls were selected from healthy individuals without any GI symptoms or positive familial or personal history of any significant disorders. The study protocol was approved by the Ethics Committee of Research Center for Gastroenterology and Liver Diseases, and a written informed consent was obtained from each participant.

Genomic DNA was extracted from peripheral blood leukocytes by standard phenol–chloroform method [27]. All exons and flanking intronic sequences of the *NOD2* gene (HUGO Gene Nomenclature Committee accession number AF178930) were amplified by previously reported primers (Table 1) [12]. A pair of primers was used for each exon except for exon 4, for which five pairs of primers were utilized, resulting in five overlapping fragments.

Polymerase chain reactions (PCRs) were performed in a 25-μl volume containing 10 mM Tris–HCl, pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 250 mM dNTPs, a 0.50-mM

Table 1 Primers used for amplification of *NOD2* exons

Screened exon	Primer		Size of PCR product (bp)	TM (°C)
	Forward	Reverse		
1	GTAGACAGATCCAGGCTCACC	CCCAGAAACAGAGTCAGCAC	293	56
2	ACCCTGCATCTGGCTTCTG	CCTTTCCTGAGAACTCTGTG	555	56
3	ACATTGCTCCATCAGCCTTC	GACTGCCCTTCCCTTTCTG	201	56
4a	TGCCTCTTCTTCTGCCTTCC	AGTAGAGTCCGCACAGAGAG	422	56
4b	TTTCTCTTTGTCTTCCCATTC	CCCTGTTCAGAGAAGCCC	380	56
4c	GAAGTACATCCGCACCGAG	AGCCAAGAGAAATGTCATCAG	446	56
4d	ATGTGCTGCTACGTGTTCTC	CAGACACCAGCGGGCACAG	456	56
4e	ACCTTCAGATCACAGCAGCC	GCTCCCCCATACCTGAAC	494	56
5	TTGTCTTACTAGCTCCATTTTC	AGCCCATTGTCCACAGCC	162	56
6	CTGTTTGCATGATGGGGGG	GGGAGATCACAGCATTAGAG	372	56
7	CGTCCCGCTGCCCCTTTC	ACTCTCTCCCTGGCTTGTC	435	56
8	AAGTCTGTAATGTAAAGCCAC	CCCAGCTCCTCCCTCTTC	380	56
9	CTTTCCCTGCTCTGACATAC	CCCCAGAGCAGAGAATCC	156	56
10	GCTGCAATGGAGAGTGGG	CTTTATTGGTTACCTTCACTTC	654	56
11	CTCACCATTGTATCTTCTTTTC	GAATGTCAGAATCAGAAGGG	228	56
12	TAAAAACAGCCCTGACTTCC	AAACTCACAGCCTGCTCAC	279	56

concentration of each primer, 2 U of Taq DNA polymerase (Fermentas, Germany), and 200 ng of genomic DNA. The PCR condition for all fragments was as follows: initial denaturation at 94°C for 10 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 10 min. Appropriate synthesis of PCR products was confirmed by agarose gel electrophoresis (1.5%) and visualized by ethidium bromide staining (0.5 μg/ml). The fragments were analyzed by an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster, CA, USA) sequencer. The sequence data were aligned using Lasergene software, version 6.00 (DNASTAR Inc., Madison, WI, USA) and were compared to the published *NOD2* gene sequence (GenBank accession number AF178930). In silico predictions were evaluated for novel coding variants using SNAP online software (http://cubic.bioc.columbia.edu/services/SNAP/). Statistical analysis was performed using X^2 test and the fisher exact test to compare variables among study groups. *P* values less than 0.05 were considered significant. The data were analyzed using SPSS software version 13 (SPSS, Chicago, IL, USA).

Results

The median age (range) of healthy controls and CD patients were 31 (17–62 years) and 32 years (16–65 years), respectively. The male to female ratio was 1.6:1.0 in both study groups. The clinical characteristics of patients based on Vienna classification [26] are summarized in Table 2.

A total of 420 chromosomes of the *NOD2* gene from 90 CD patients and 120 healthy controls were screened for any exonic nucleotide changes by direct sequencing. In analyzed coding regions and intronic flanking sequences, 21 variations were detected in four exons including exons 2, 4, 8, and 11. The identified mutations are listed in Table 3 in comparison to previously reported variations in the *NOD2* gene sequence by Lesage et al. [12]. Splice sites and other exons did not demonstrate any sequence change in our study population. The observed sequence changes were mostly concentrated in exon 4, which included higher than 70% (n=16) of the detected variations. Among five remaining mutations, four were observed in exons 2 and 8, two in each and one in exon 11. No insertion or deletion mutations were detected.

Table 2 Clinical characteristics of Iranian patients with Crohn's disease (CD)

Crohn's disease patients (n=90)[a]	
Disease location	
Ileal (L1)	27 (30%)
Colonic (L2)	27 (30%)
Ileocolonic (L3)	33 (36.6%)
Upper GI (L4)	3 (3.3%)
Disease behavior	
Inflammatory (B1)	52 (57.7%)
Stricturing (B2)	18 (20%)
Penetrating (B3)	20 (22.2%)
Age at diagnosis (under 40 years)	37 (41.1%)

[a] Based on Vienna classification [26]

Most variations were a transition type (61.9%), especially a C→T substitution occurred more frequently (33.3%). All observed variations were due to a single nucleotide substitution, except the insertion frameshift mutation, 1007fsinsC (SNP13). A novel nonsense mutation was detected in exon 4, which resulted from a C to A transversion forming a premature stop codon in the location 813 of the amino acid chain. More than 50% (n=11) of the observed variations were missense mutations.

Over 85% (n=18) of the observed variations had an allele frequency of more than 1% in CD patients, while only 47.6% (n=10) of these mutations showed the same frequency in controls. Eight sequence changes had an allele frequency of more than 5% in CD patients including S178S, P268S, P371T, R459R, R587R, R702W, A794P, and Q809H (Figs. 1 and 2). Four of them had a frequency of more than 10% in healthy controls, including S178S in exon 2 and P268S (SNP5), R459R (SNP6), and R587R (SNP7) in exon 4. None of the four mentioned mutations showed a significant higher frequency in CD patients compared to controls.

Totally, eight novel variations were identified in Iranian CD patients, one in exon 2 and seven in exon 4 (Fig. 3). Five out of eight were missense mutations, of which three of them, including P371T, A794P, and Q809H, had a higher allele frequency in CD patients compared to controls (P< 0.001, with odds ratio (OR)=9.26, 7.07, and 9.26, respectively). The related 95% confidence intervals were as follows: P371T, OR=9.26, 95% CI, 2.06–41.59; A794P, OR=7.71, 95% CI, 2.21–26.88; and Q809H, OR=9.26, 95% CI, 2.06–41.59. None of these novel variations presented with an allele frequency of more than 10% in healthy controls.

Cumulative risk was performed for eight rare mutations including V162I, N289S, T389T, V432A, E462K, A611A, S732S, and R760C. The number of CD patients carrying rare variants was significantly higher compared to controls (17.8% in CD patients vs. 0.8% in controls, P<0.001, OR= 25.73, 95% CI, 3.34–198.08).

In silico predictions for coding novel variants—using SNAP online software (http://cubic.bioc.columbia.edu/services/SNAP/)—are presented in Table 4. The power calculation was performed for variations without a

Table 3 *NOD2* variations detected in 90 Iranian CD patients and 120 healthy controls

Location and nucleotide change	dbSNP accession number	Peptide change	NCBI's (buid36) position	Polymorphic marker [a]	Protein domain	No. (%) of variant alleles		*p* value	No. (%) of variant alleles in a European population [b]		
						CD (*n*=180)	Controls (*n*=240)		CD (*n*=906)	UC (*n*=318)	Control (\n=206)
Exon 2:											
484 G→A	EF364441	V162I			CARD1	3 (1.7)	0 (0)	NS	0	0	0
534 C→G	Rs2067085	S178S	49291360		CARD2	53(29.4)	70 (29.2)	NS	280(31)	79 (25)	68 (33)
Exon 4:											
802 C→T	Rs2066842	P268S	49302125	SNP5		62 (34.4)	71 (29.6)	NS	375(41)	77 (24)	57 (28)
866 A→G	Rs5743271	N289S	49302189		NBD	2 (1.1)	1 (0.4)	NS	6	0	3
1111 C→A	EF624393	P371T			NBD	10 (5.5)	2(0.8)	<0.001[c]	0	0	0
1167 G→A	EU817180	T389T			NBD	1 (0.6)	0 (0)	NS	0	0	0
1295 T→C	Rs2076754	V432A	49302618		NBD	1 (0.6)	0 (0)	NS	1	0	0
1377 T→C	Rs2066843	R459R	49302700	SNP6	NBD	62 (34.4)	68(28.3)	NS	379(42)	76 (24)	59 (29)
1384 G→A	EF624392	E462K			NBD	2 (1.1)	0 (0)	NS	0	0	0
1761 T→G	Rs1861759	R587R	49303084	SNP7		46 (25.6)	65 (27.1)	NS	301(33)	142(45)	83 (40)
1833 C→T	EF624394	A611A				3 (1.7)	0 (0)	NS	3	6	5
2104 C→T	Rs2066844	R702W	493030427	SNP8		24 (13.3)	3(1.3)	<0.001[d]	98 (11)	10 (3)	9 (4)
2196 C→T	Rs6413461	S732S	49303519			2 (1.1)	0 (0)	NS	0	0	0
									0	0	0
2278 C→T	Rs3813758	R760C	49303601		LRR	2 (1.1)	0 (0)	NS	0	0	0
2376 C→T	Rs5743280	P792P	49303699		LRR	8 (4.4)	3 (1.3)	NS	0	0	0
2380 G→C	EU888273	A794P			LRR	16 (8.9)	3 (1.3)	<0.001[e]	0	0	0
2427 G→C	EU888274	Q809H			LRR	13 (7.2)	2 (0.8)	<0.001[f]	0	0	0
2439 C→A	EU888275	C813X			LRR	4 (2.2)	7 (2.9)	NS	0	0	0
Exon 8:											
2718 C→T	Rs58586167	F906F	49314037		LRR	1 (0.6)	4 (1.7)	NS	0	0	0
2722 G→C	Rs2066845	G908R	49314041	SNP12	LRR	4 (2.2)	3 (1.3)	NS	55 (6)	1 (.3)	2 (1)
Exon 11:											
3020insC	Rs5743293	1007fs	49321282:49321283	SNP13	LRR	3 (1.7)	2 (0.8)	NS	96 (11)	4 (1)	4 (2)

NS not significant

[a] The correspondence with the polymorphic sites described by Hugot et al. [7]

[b] According to a study in Europe by Lesage et al. [12]

[c] OR=9.263; 95% CI, 2.063–41.590

[d] OR=12.154; 95% CI, 3.599–41.049

[e] OR=7.707; 95% CI, 2.210–26.877

[f] OR=9.263; 95% CI, 2.063–41.590

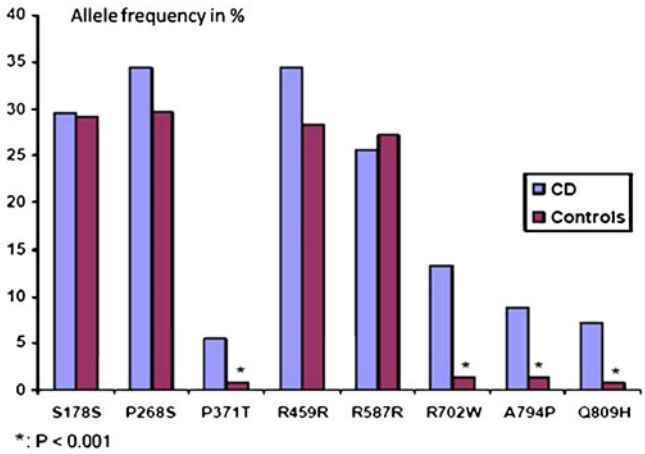

Fig. 1 The allele frequency of the eight most frequent variants of the *NOD2* gene in Iranian patients with Crohn's disease and healthy controls

significant *p* value. The power for these analyses varied from 5.5% in S178S to 61.7% in P792P, which could be due to the small number of patients included in the study.

Among the three common mutations of the *NOD2* gene, only R702W (SNP8) showed a significantly higher allele frequency in CD patients compared to healthy controls (13.3% vs. 1.3%, $P<0.001$, OR=12.15, 95% CI, 3.60–41.05). The allele frequencies of SNP 12 (G908R) and SNP 13 (1007fsinsC) were lower than 3% in Iranian CD patients (2.2% and 1.7%, respectively).

Discussion

This study provides confirmation for the previously reported [20] association of the NOD2 R702W mutation with CD in Iranian population ($p<0.001$). In addition, we observed eight novel variations in exonic regions of the *NOD2* gene. Finally, allele frequency analysis among patients and healthy controls indicated the association of some of the *NOD2* gene variations with CD in Iranian population.

The *NOD2* gene is known to be involved in susceptibility to CD in many Caucasian studies. Three common variants of this gene (G908R, R702W, and 1007fsinsC) were documented to be strongly associated with CD in white American and European populations [28–30]. Analysis of non-Caucasian groups has failed to identify a significant contribution of these variations in CD susceptibility. In Japanese and Korean populations, as well as in Chilean patients, the *NOD2* common variants were very rare [30–32]. Iranian population, in the Middle East, has a complete mixed genetic reservoir. During the past years, some ethnicities like Turkish and Arabs who were mixed

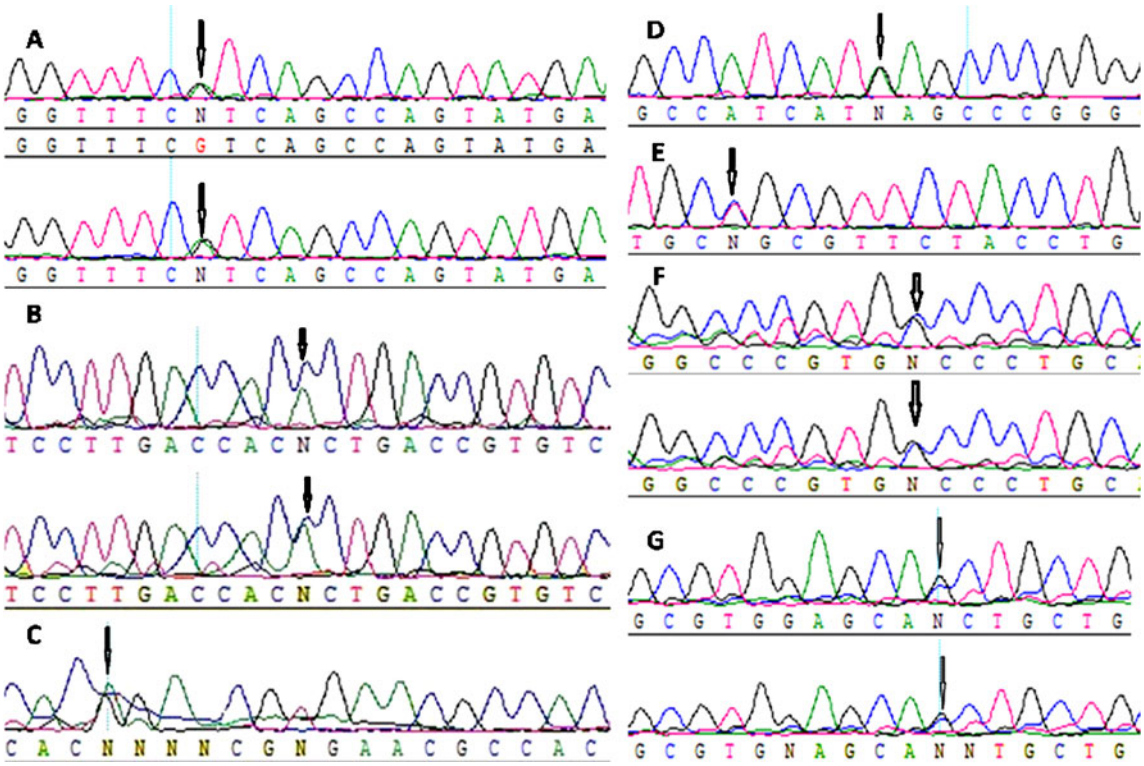

Fig. 2 Forward and reverse electropherograms of *NOD2* gene. **a** V162I variation in a CD patient, with a heterozygote genotype; **b** P371T variation in a CD patient, with a heterozygote genotype; **c** T389T variation in a CD patient, with a heterozygote genotype; **d** E462K variation in a CD patient, with a heterozygote genotype; **e** A611A variation in a CD patient, with a heterozygote genotype; **f** A794P variation in a CD patient, with a heterozygote genotype; **g** Q809H variation in a CD patient, with a heterozygote genotype

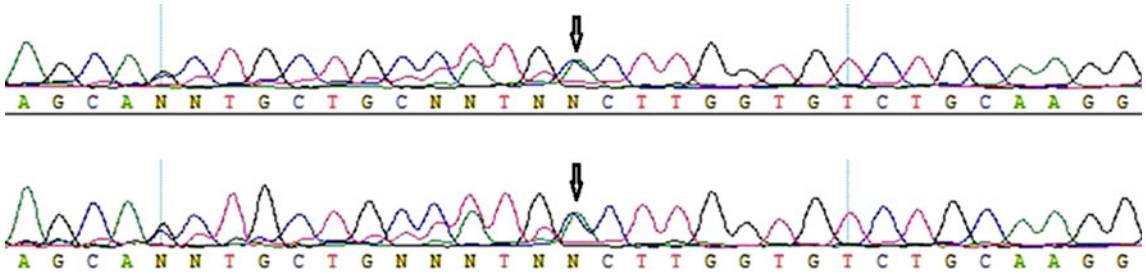

Fig. 3 Forward and reverse electropherograms of C813X variation in a CD patient, with a heterozygote genotype

with the inhabitant population previously immigrated to Iran from northern regions. This makes Iranians have a different genetic background in comparison to Caucasians as well as Asians. In a recent study in Iranian CD patients, only R702W (SNP8) was reported to be associated with CD ($p<0.001$) [20]. The different distribution patterns of the *NOD2* common variations raised an opinion whether any other sequence changes of this gene were present in CD patients in Iran.

In this study, 21 variations were detected, including eight novel mutations, comparing to dbSNP sequence. Many studies have attempted to discover other sequence variations of the *NOD2* gene in different populations [12, 33]. In a multi-center study on European population, 67 variations were identified in 453 CD cases [12], while in another research in Scotland, 18 mutations were reported in 663 CD patients [33]. According to these results, the *NOD2* sequence changes in Iran showed a comparable occurrence and frequency to European studies.

More than 70% ($n=16$) of the observed variations occurred in exon 4, a 1,816-bp fragment, which encodes a large size of the NOD2 protein from NBD to LRR segment. LRR fragment of the NOD2 protein has a critical role in the detection of bacterial components [11]; thus, sequence changes influencing this part of the protein could probably result in an impaired function.

Three of the novel variations (P371T, A794P, and Q809H) showed a statistically significant higher frequency in CD patients compared to controls ($P<0.001$). These variations were all in a conserved sequence of the NOD2 gene, assessed by UCSC website (http://genome.ucsc.edu). The observed associations could clarify the probable role of the *NOD2* gene variations in susceptibility to CD in Iran. As a separate ethnicity, Iranian patients demonstrated a different occurrence of *NOD2* variations.

Two common mutations of the *NOD2* gene (G908R and 1007fsinsC) were previously reported with a frequency lower than 5% in Iranian CD patients without any significant association with disease [20]. These results were in line with our observations. The allele frequencies of SNP 12 (G908R) and SNP 13 (1007fsinsC) were presented, 2.2% and 1.7%, respectively in CD patients. None of these variations showed a significant higher frequency in CD patients compared to controls. These results are in contrast to the reports from other Caucasian populations with a high frequency of these variations [28–30].

The analysis method used in this study did not include the promoter or other potential regulatory sequences which may influence the gene expression; and also, the number of included cases was somehow restricted. Despite these limitations, a comparable number of variations were detected in exonic regions of the *NOD2* gene in Iranian CD patients.

Table 4 In silico predictions for coding novel variants of NOD2 gene using SNAP online software (http://cubic.bioc.columbia.edu/services/SNAP/) with reliability index and expected accuracy

dbSNP accession number	Peptide change	Prediction	Reliability index	Expected accuracy
EF364441	V162I	Non-neutral	1	63%
EF624393	P371T	Neutral	3	78%
EU817180	T389T	Neutral	1	60%
EF624392	E462K	Neutral	1	60%
EF624394	A611A	Neutral	2	69%
EU888273	A794P	Non-neutral	3	78%
EU888274	Q809H	Neutral	2	69%

Reliability indices are indicative of confidence in prediction. Expected accuracy is a number of correctly predicted (at a given reliability index) neutral or non-neutral samples in the SNAP testing set. This measure of accuracy is meant to illustrate the likelihood that a given prediction is correct

The observed variations, especially missense mutations (n=4), could theoretically change the expression and function of the NOD2 protein. Changes in the structure of this protein may account as a predisposing factor for CD.

In conclusion, our study demonstrated the association between some of the *NOD2* variations with CD in Iranian patients and could propose this gene as a contributing genetic factor in disease susceptibility. Evaluation of exonic and non-exonic segments including promoter regions in a study with larger sample size would be helpful in establishing the exact role of the *NOD2* gene in Iranian CD patients.

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