Original Article

Detection of mutations in *NOD2/CARD15* gene in Arab patients with Crohn's disease

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Abstract

Background: Mutations in *NOD2/CARD15* gene have been linked to an increased risk of Crohn's disease (CD). The objective of this study is to determine *NOD2/CARD15* gene mutations, and their association with the risk of CD in Arabs in Kuwait.

Methods: Four *NOD2* gene mutations, including Pro268Ser (SNP5), Arg702Trp (SNP8), Gly908Arg (SNP12), and Leu1007FsinsC (SNP13) were examined in Arab CD patients (n = 103) and control subjects (n = 100). The genomic DNA was isolated and used in polymerase chain reaction (PCR) with four sets of specific primers. The PCR-amplified DNA fragments were sequenced and analyzed for the *NOD2* mutations. Logistic regression was used to estimate the adjusted odds ratios (aOR) and 95% confidence intervals (CI).

Results: Of the four genotyped variants, the Arg702Trp (SNP8) and Leu1007FsinsC (SNP13) variants were not informative in our study sample due to minor allele frequency of <1%. The Pro268Ser (SNP5) mutation was detected in 17 (16.5%) CD patients and 32 (32.0%) controls. The Gly908Arg (SNP12) mutation was observed in 24 (23.3%) patients and 10 (10.0%) controls. In the dominant genetic risk model (i.e. carrying at least one minor allele), CD patients compared to controls were less likely to carry either the "CT" or "TT" genotype of variant Pro268Ser (SNP5; aOR = 0.43, 95% CI: 0.22–0.84). In contrast, CD patients compared to controls were more likely to carry the homozygous for the minor allele or the heterozygous genotypes of variant Gly908Arg (SNP12; aOR = 2.67, 95% CI: 1.19–5.97).

Conclusions: In this Arab population, carrying at least one copy of the minor allele of Gly908Arg (SNP12) mutation in *NOD2* gene was associated with increased susceptibility to CD, while having the heterozygous or homozygous for the minor allele genotype of the Pro268Ser (SNP5) mutation provided protection against CD. Mutations in Arg702Trp (SNP8) and Leu1007FsinsC (SNP13) were not detected in this sample of the Arab population in Kuwait.

Keywords: Arab, *CARD15*, Crohn's disease, genetic, Kuwait, *NOD2*

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INTRODUCTION

The etiology of Crohn's disease (CD) is not precisely known, but it is believed to occur due to interaction between genetic and environmental factors along with alterations in immune regulatory mechanisms.[1] An abnormal inflammatory response against the normal enteric microflora in a genetically susceptible host is well established, suggesting an underlying inflammatory dysregulation against gut microflora.[1] Therefore, most CD-associated genes investigated so far are those involved in inflammation and immune responses. A recent integrative analysis of inflammatory bowel disease (IBD) genome-wide association studies analyzed nearly 60,000 subjects (including more than 25,000 cases of IBD) and identified approximately 240 loci statistically associated with the risk of developing IBD.[2] The nucleotide-binding oligomerization domain 2 (*NOD2*) gene, also known as the caspase recruitment domain-containing protein 15 (*CARD15*) gene, is present in the IBD1 region.[3] Due to its role in recognizing bacterial components by the host cells, *NOD2* has been investigated as a susceptibility gene in CD and plays an important role in the development of innate immunity.[4] Although, the *NOD2* gene is the most studied IBD gene, so far reports have produced conflicting data with regards to the percentage of mutations reported (<50% in CD patients).[5] These mutations are suggested to influence the onset, severity, complications, prognosis, and effectiveness of treatment of CD.[6,7]

The three most studied *NOD2* mutations, alleles Arg702Trp (SNP8) (C2104T, R702W), Gly908Arg (SNP12) (G2722C, G908R), and Leu1007FsinsC (SNP13) (3020insC, 1007fs) are significantly associated with susceptibility to CD in the American and European Caucasian populations.[8,9] However, the above mutations are remarkably heterogeneous in different populations, with variation across Europe and other parts of the world.[10] Genotype-phenotype analyses demonstrate an association of these mutations with ileum-specific symptoms and an increased incidence of the fibrostenotic phenotype.[11] In addition, to the three above described major mutations in CD patients, a fourth major mutation, Pro268Ser (SNP5) (C802T, P268S), has been described in North Indians, Zhuang Chinese, and Malaysians.[12-14] The presence of Pro268Ser (SNP5) in the absence of known *NOD2* mutations was correlated with increasing age and adult-onset of CD.[15] The Pro268Ser (SNP5) gradient between Africa and the Middle East and its absence in Asian and Native American populations indicated that the evolution of this variant occurred in the Middle East.[16]

There is no report on the role of *NOD2* mutations in CD in the Arab population. Therefore, the aim of this study was to find the frequency of the four major *NOD2* mutations [Pro268Ser (SNP5), Arg702Trp (SNP8), Gly908Arg (SNP12), and Leu1007FsinsC (SNP13)] in Kuwaiti Arab patients with CD (cases), and non-IBD subjects (controls); and determine their associations with the presence of CD and clinical characteristics of the disease, such as age at the time of diagnosis, disease location, and behavior, as well as the presence of extraintestinal manifestations and family history.

PATIENTS AND METHODS

Selection of subjects

Adult (age ≥ 21 years) Kuwaiti Arab patients with an established diagnosis of CD were invited to participate in the study. The diagnosis of CD was established on the basis of clinical presentation and supported by appropriate laboratory investigations, typical findings on radiological studies, colonoscopy, upper endoscopy, or surgery, and confirmed by histopathology.[17] The controls were a group of non-IBD patients recruited from the same hospital. The medical records of the CD patients were reviewed to obtain the following data: demographics (age, gender, nationality, smoking), age at diagnosis, family history, disease location, and behavior and presence of extraintestinal manifestation. The age at the time of diagnosis (A), location (L), and behavior (B) of disease was determined using the Montreal Classification [Table 1].[18] The location (L) and behavior (B) of the disease were determined on the basis of the most recent clinical assessment, endoscopic and radiologic investigations, and surgical notes available at the time of inclusion in the study.

Ethical considerations and declaration of patient consent

The study was approved by the Ethical Committee of the Health Sciences Center, Kuwait University, as well as

Table 1: The Montreal Classification of Crohn's disease*

Criteria	Group	Definition
Age at diagnosis (A)	A1	<16 years
	A2	17-40 years
	A3	>40 years
Location (L)	L1	Ileal
	L2	Colonic
	L3	Ileo-colonic
	L4†	Isolated upper disease
Behavior (B)	B1	Non-stricturing, non-penetrating
	B2	Stricturing
	B3	Penetrating
	p‡	Perianal

* According to the Montreal Classification.[18]. †L4 is a modifier that can be added to L1-L3 when concomitant upper gastrointestinal disease is present. ‡ *P* is added to B1-B3 when concomitant perianal disease is present

the Standing Committee for Coordination of Health and Medical Research of the Ministry of Health, Kuwait. The authors certify that they obtained all appropriate patient consent forms. In the form the patient(s) gave his/her consent for his/her clinical information to be reported in the journal. The patients understood that their names and initials will not be published, and due efforts will be made to conceal their identity.

Genomic DNA isolation

Genomic DNA was isolated from the peripheral blood samples using Qiagen kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The deoxyribonucleic acid (DNA) concentration in each sample was adjusted to 1 mg/mL, aliquoted, and stored at −80°C until use.

NOD2 allele selective primer design and synthesis

The forward and reverse primers [Table 2] specific for the region covering the test single nucleotide polymorphisms (SNPs), were designed based on the available *NOD2* gene sequence (accession no. NG_007508.1), or were taken from the published work, and authenticated by the Primer Design Software (https://www.dnastar.com/t-sub-solutions-molecular-biology-primer-design.aspx).[12,13] These primers were synthesized in-house using a 3400 DNA Synthesizer (Applied Biosystems, Foster City, CA, USA).

PCR amplification of the target regions

The target regions were amplified by PCR using the forward and reverse primers (25 pmol each), genomic DNA (100 ng), deoxynucleotide triphosphates (250 μM), tris hydrochloric acid (10 mM, pH 8.3), potassium chloride (50 mM), magnesium chloride (2 mM), and 2.5 units of *AmpliTaq* Gold® DNA polymerase (Perkin-Elmer Applied Biosystems). PCR cycles were performed with an initial denaturation step of 10 min at 95°C, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final extension step of 72°C for 5 min. All the primer pairs were tested to amplify the target DNA of the expected size with randomly selected genomic DNA samples. The results showed that all primer pairs amplified the target of expected sizes. This showed that all primers were appropriate to conduct further experiments. However, to avoid duplications, only one set of primers were used in further experiments to detect each type of mutation [Pro268Ser (SNP5): F1.1 and R1.1; Arg702Trp (SNP8): F2.1 and R2.1; Gly908Arg (SNP12): F3.1 and R3.1; and Leu1007FsinsC (SNP13): F4.1 and R4.1].

Sequencing of PCR products

The PCR products were purified using a High Pure PCR Product Purification Kit (Roche Molecular Diagnostics, Pleasanton, CA, USA). The purified PCR DNA fragment (10 ng) was used in the cycle-sequencing reaction using a BigDye Terminator v1.1 Cycle-Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA), and the reaction products were analyzed using an ABI 3130x Genetic Analyzer (Thermo Fisher Scientific). The obtained sequences were compared with the *NOD2* gene sequence (GenBank accession number AF178930), using Pairwise Sequence Alignment (NUCLEOTIDE) using EMBOSS Needle (European Molecular Biology Laboratory, Heidelberg, Germany). All the PCR products were sequenced to identify the mutations. The two representative electropherograms of the sequence obtained are shown in Figures 1 and 2.

Statistical analysis

The statistical analysis was performed using IBM SPSS Statistics software (IBM, Armonk, NY, USA) and SAS 9.4 (SAS Institute, Cary, NC, USA). The statistical significance level was set to $\alpha = 0.05$ for all association analyses. Descriptive analyses were conducted to calculate frequencies and proportions of categorical variables and means and standard deviations (SD) of continuous variables. Genotype frequencies of the analyzed genetic variants were determined for cases and controls. Chi-square (χ^2) tests were

Table 2: The details of NOD2 single nucleotide polymorphisms (SNPs), nucleotide sequences of the primers, the primer annealing sites in the gene sequence (accession no. NG_007508.1, length: 42938 bp) and the expected PCR product sizes

SNP designations	Location/ Mutations	Amino acid substitutions	Primers' specifications		PCR product sizes (bp)
			Nucleotide sequences (5'-3')	Primer annealing sites in the gene	
rs2066842 (SNP5)	Exon4/802C>T		*F1.1 gctgccacatgcaagaagta	18419-18438	396
			R1.1 agtccgcacagagagtggtt	18814-18795	
rs2066844 (SNP8)	Exon4/2104C>T	Arg702Trp	F2.1 caccagctttgctcagacac	19638-19657	392
			R2.1 ctcctcctgcatctcgtaca	20029-2010	
rs2066845 (SNP12)	Exon8/2722G>C	Gly908Arg	F3.1 gtgaggccactctgggatt	30350-30368	213
			R3.1 ccacctcaagctctggtgat	30562-30543	
rs2066847 (SNP13)	Exon11/3020insC	Leu1007FsinsC	F4.1 gacaggtgggcttcagtaga	35571-37590	283
			R4.1 attctgccattcctctctcc	37853-37384	

* F: forward, R: reverse. The sequences of primers F1.1, R1.1, F2.1, R2.1, F3.1, R3.1, F4.1 and R4.1 are according to Juyal, *et al*.[12] and Long, *et al*.[13]

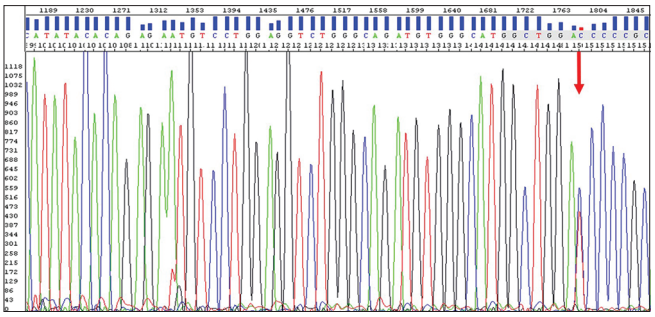

Figure 1: The sequence and corresponding electropherogram for patients with Crohn's disease. Here the mutation is at rs2066842 [Pro268Ser (SNP5), Exon4 802C > T], and as a result peak corresponding to T and C overlaps

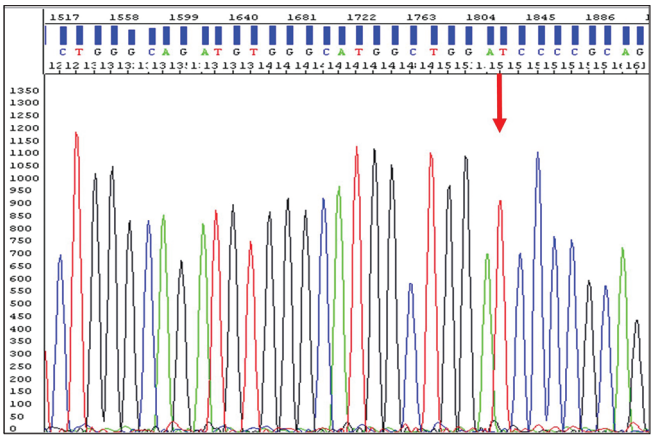

Figure 2: The sequence and corresponding electropherogram for the Crohn's disease patients. Here the mutation is at rs2066842 [Pro268Ser (SNP5), Exon4 802C > T], and as a result peak corresponding to C is replaced by T

used to assess associations between categorical variables. Deviation from Hardy–Weinberg Equilibrium (HWE) was tested for the polymorphic variants (i.e. variants with minor allele frequency >1%) in the total study sample, among cases and controls using goodness-of-fit χ^2 tests. A *P* value <0.05 was used as an indicator for possible deviation from HWE. Associations between genetic variants and the presence of CD were assessed using co-dominant and dominant genetic risk models. Logistic regression was applied to estimate odds ratios (OR) and their 95% confidence intervals (CI). Unadjusted ORs and adjusted ORs (aOR) were estimated. Adjusted models accounted for the effect of gender.

RESULTS

The sociodemographic characteristics of the patients and controls are shown in Table 3. A total of 103 Arab patients (54 males; 49 females) with CD, and 100 healthy control subjects (34 males; 66 females) were included in the study. The mean age of the patients and the controls at the time of inclusion in the study was 33.4 ± 9.8 (range 21–64) and 31.5 ± 7.5 (range 22–62) years, respectively. The mean age of the patients at the time of diagnosis of CD was 23.3 ± 9.6 (range 6–54) years. The disease was localized to the distal small intestine, with or without the involvement of the cecum (L1) in 21 (20.4%) patients. Only the colon (L2) was affected in 35 (34.0%) patients and the involvement of both ileum and colon (L3) was found in 47 (45.6%) patients. The behavior of CD was non-stricturing, non-penetrating (B1) in 57 (55.3%) patients, while stricturing (B2) and penetrating (B3) disease was found in 25 (24.3%) and 21 (20.4%) patients, respectively. Perianal disease was present in 13 (12.6%) patients. Family history of CD and ulcerative colitis was present in a first- or second-degree relative in 22 (21.4%) and 1 (1.0%) patients, respectively. A total of 21 (20.4%) patients had at least one extraintestinal manifestation of CD.

Cases and controls were genotyped for *NOD2/CARD15* variants Pro268Ser (SNP5), Arg702Trp (SNP8), Gly908Arg (SNP12), and Leu1007FsinsC (SNP13). Variants Arg702Trp (SNP8) and Leu1007FsinsC (SNP13) were not informative in our study sample due to minor allele frequency <1% [Table 4], and hence, were excluded from the analysis. The analyzed variants [Pro268Ser (SNP5) and Gly908Arg (SNP12)] demonstrated deviation from the HWE.

Table 5 shows the results of the association analysis between variants Pro268Ser (SNP5) and Gly908Arg (SNP12) and the presence of CD. In the co-dominant genetic risk model, CD patients were less likely than controls to have the heterozygous genotype (aOR = 0.42, 95% CI: 0.19–0.94) and the homozygous for the minor allele genotype (aOR = 0.45, 95% CI: 0.15–1.31; this association did not gain statistical significance) of variant Pro268Ser (SNP5). In the dominant genetic risk model (i.e. carrying at least one minor allele), CD patients compared to controls were less likely to carry either the "CT" or "TT" genotype of variant Pro268Ser (SNP5; aOR = 0.43, 95% CI: 0.22–0.84). In contrast, CD patients compared to controls were more likely to carry the homozygous for the minor allele or the heterozygous genotypes of variant Gly908Arg (SNP12; aOR = 2.67, 95% CI: 1.19–5.97).

Table 6 shows bivariate associations of Pro268Ser (SNP5) and Gly908Arg (SNP12) mutations with the age at the time of diagnosis of CD, location and behavior of the disease, family history, and presence of extraintestinal manifestation. There was no difference in the mean age at the time of diagnosis in patients with and without Pro268Ser (SNP5) mutation (24.53 ± 9.2 and 23.0 ± 9.7 years, respectively).

Table 3: Sociodemography and clinical characteristics of CD patients and controls included in the study

Sociodemography and clinical characteristics	CD Patients		Controls	
	n	(%)	*n*	(%)
Gender				
Male	54	(52.4)	34	(34)
Female	49	(47.6)	66	(64)
Current Age (years)				
Mean±SD (range)	33.4±9.8 (21-64)		31.5±7.5 (22-62)	
Age at diagnosis*				
Mean±SD (range)	23.3±9.6 (6-54)		-	
<16 years (A1)	34	(33.0)	-	-
17-40 years (A2)	62	(60.2)	-	-
> 40 years (A3)	7	(6.8)	-	-
Location of disease*				
Ileal (L1)	21	(20.4)	-	-
Colonic (L2)	35	(34.0)	-	-
Ileo-colonic (L3)	47	(45.6)	-	-
Behavior of disease*				
Non-stricturing, non-penetrating (B1)	57	(55.3)	-	-
Stricturing (B2)	25	(24.3)	-	-
Penetrating (B3)	21	(20.4)	-	-
Perianal disease (p)	13	(12.6)	-	-
Family History				
Crohn's disease	22	(21.4)	1	(1.0)
Ulcerative colitis	1	(1.0)	0	(0.0)
Extraintestinal manifestations				
Skeletal	16	(15.5)	-	-
Oral ulcers	4	(3.9)	-	-
Eye	1	(1.0)	-	-

* According to the Montreal Classification.[18]

Similarly, there was no difference in the mean age at diagnosis for patients with and without Gly908Arg (SNP12) mutation (22.4 ± and 23.5 ± 9.9 years, respectively). Patients with Gly908Arg (SNP12) mutation compared to those without the mutation had higher rates of disease of ileum and colon (L3; 50.0% vs. 29.1%) and lower rates of colonic disease (L2; 4.2% vs. 25.3%, *P* value = 0.033). There was no significant difference in the behavior of the disease, family history of IBD, or presence of extraintestinal manifestations in patients with or without Pro268Ser (SNP5) or Gly908Arg (SNP12) mutation.

DISCUSSION

This is the first study to examine the frequency of four *NOD2* mutations [Pro268Ser (SNP5) (C802T, P268S), Arg702Trp (SNP8) (C2104T, R702W), Gly908Arg (SNP12) (G2722C, G908R), and Leu1007FsinsC (SNP13) (3020insC, 1007fs)] in CD patients (cases), and non-IBD subjects (controls), in an Arab population. We found that Pro268Ser (SNP5) (C802T, P268S) mutation occurs more frequently in controls compared to patients. This is consistent with studies from

Table 4: Genotype and minor allele frequencies and Hardy-Weinberg Equilibrium test for the analyzed variants in the total study sample (*n*=203), among cases (*n*=103), and among control subjects (*n*=100)

Variant	Genotypes	Genotype frequencies, *n*	Minor allele frequency	HWE *P**
Total sample				
rs2066842 (SNP5)	CC/CT/TT	154/33/16	0.16	<0.001
rs2066844 (SNP8)†	CC/CT/TT	202/1/0	0.003	NA
rs2066845 (SNP12)	GG/GC/CC	169/24/10	0.11	<0.001
rs2066847 (SNP13)†	Wild type/mutant	203/0	0	NA
Cases				
rs2066842 (SNP5)	CC/CT/TT	86/11/6	0.11	<0.001
rs2066844 (SNP8)†	CC/CT/TT	102/1/0	0.005	NA
rs2066845 (SNP12)	GG/GC/CC	79/14/10	0.17	<0.001
rs2066847 (SNP13)†	Wild type/mutant	103/0	0	NA
Controls				
rs2066842 (SNP5)	CC/CT/TT	68/22/10	0.21	0.003
rs2066844 (SNP8)†	CC/CT/TT	100/0/0	0	NA
rs2066845 (SNP12)	GG/GC/CC	90/10/0	0.05	1.00
rs2066847 (SNP13)†	Wild type/mutant	100/0	0	NA

HWE: Hardy-Weinberg Equilibrium; NA: not applicable. † These variants were not analyzed due to their minor allele frequency being<1%. * *P* is testing the hypothesis that variants are in HWE

Table 5: Associations between genetic variants rs2066842 (SNP5) and rs2066845 (SNP12) genotypes and the presence of Crohn's disease, using co-dominant and dominant genetic risk models

Variant	Genetic Model/ Genotypes	Cases (*n*=103), % (*n*)	Controls (*n*=100), % (*n*)	Unadjusted OR (95% CI)	*P*	Adjusted OR* (95% CI)	*P*
Pro268Ser (SNP5)	Co-dominant						
	CC	83.5 (86)	68.0 (68)	1.00 (Reference)	-	1.00 (Reference)	-
	CT	10.7 (11)	22.0 (22)	0.40 (0.18-0.87)	0.021	0.42 (0.19-0.94)	0.034
	TT	5.8 (6)	10.0 (10)	0.47 (0.16-1.37)	0.168	0.45 (0.15-1.31)	0.141
	Dominant						
	CC	83.5 (86)	68.0 (68)	1.00 (Reference)	-	1.00 (Reference)	-
	CT+TT	16.5 (17)	32.0 (32)	0.42 (0.22-0.82)	0.011	0.43 (0.22-0.84)	0.014
Gly908Arg (SNP12)	Co-dominant						
	GG	76.7 (79)	90.0 (90)	1.00 (Reference)	-	1.00 (Reference)	-
	GC	13.6 (14)	10.0 (10)	1.60 (0.67-3.79)	0.291	1.47 (0.61-3.56)	0.392
	CC	9.7 (10)	0.0 (0)	NE	-	NE	-
	Dominant						
	GG	76.7 (79)	90.0 (90)	1.00 (Reference)	-	1.00 (Reference)	-
	GC+CC	23.3 (24)	10.0 (10)	2.73 (1.23-6.07)	0.013	2.67 (1.19-5.97)	0.018

OR: odds ratio; CI: confidence interval; NE: not estimable due to zero count. * Adjusted for sex

India where Pro268Ser (SNP5) mutation was not associated with CD.[12] Our data show that the Pro268Ser (SNP5) mutation appears to have a protective effect against CD in the Kuwaiti Arab population. Our results also suggest that Gly908Arg (SNP12) mutation occurs more frequently in Kuwaiti Arabs with CD compared to controls. This is in contrast to the study comparing the Israeli Arab population which found that Gly908Arg (SNP12) (G2722C, G908R) mutation was rare in both CD patients and controls.[10]

Epidemiological and linkage studies suggest that genetic factors play a significant role in determining CD susceptibility, and the most associated gene is *NOD2/CARD15*. Polymorphisms in the *NOD2* gene reduce NOD2/CARD15 protein function, impairing the inflammatory response to external stimuli. Several variants were identified as genetic determinants of CD susceptibility, even if with a remarkable heterogeneity among racial and geographical groups. In a comprehensive study using DNA samples from 52 worldwide populations for these four major mutations in *NOD2* gene, it has been reported that Arg702Trp (SNP8), Gly908Arg (SNP12), and Leu1007FsinsC (SNP13) are mainly Caucasian alleles, with strong distribution dissimilarity between single populations and major geographical regions.[16] This regional diversity of *NOD2* mutations within Europe points to the regional existence of selection pressure. These polymorphisms alter the structure of the protein at the level of the LRR domain or the adjacent regions, interfering with bacteria recognition and increasing the production of IL-12, IL13, IL23, and other pro-inflammatory cytokines, leading to chronic inflammation.

In Croatian CD patients, *NOD2* gene mutation rate (27.9%) is somewhat lower compared to other reports but is reported to associate with an early onset and a higher need for surgery.[19] In the Iranian population, mutations in Arg702Trp (SNP8), Gly908Arg (SNP12), and Leu1007FsinsC (SNP13) showed frequencies of 13.3, 2.2, and 1.7%, respectively which is less than reported in the Caucasian population, and only Arg702Trp (SNP8) was significantly more frequent in CD patients compared to controls.[20,21] In another study, pediatric CD patients in Iran were not found to carry these mutations.[22] A study from Morocco also reported no association of the three *NOD2* alleles with local CD patients.[23] However, in Algerian patients, Gly908Arg (SNP12) mutation was significantly more common in CD patients compared to controls, and Arg702Trp (SNP8) mutation was associated with CD outcome and early onset of disease.[24] In the Dutch patients, Gly908Arg (SNP12) and Leu1007FsinsC (SNP13) alleles were associated with CD, and at least one NOD2 mutation was reported to be associated with complications.[25] In the Indian population, Arg702Trp (SNP8), Gly908Arg (SNP12), and Leu1007FsinsC (SNP13) mutations were reported to be uncommon in CD.[26] Leu1007FsinsC (SNP13) mutation was reported in the Hungarian and Serbian adult CD patients, while in the Hungarian pediatric population Gly908Arg (SNP12) and Leu1007FsinsC (SNP13) mutations were significantly associated with an increase in the risk of CD.[8,27] A group from Malaysia reported the absence of the common *NOD2* alleles in a mixed population consisting of Malay, Indians, and Chinese CD patients.[28] Similarly, a study from Korea also reported the absence of the common alleles of NOD2 in the pediatric population.[29]

In the French population, Arg702Trp (SNP8), Gly908Arg (SNP12), and Leu1007FsinsC (SNP13) mutations with compound heterozygosity were reported to increase the risk of CD, and Arg702Trp (SNP8) was found to be the sole allele showing a significant

Table 6: Associations of sociodemography and clinical characteristics of Kuwaiti CD patients with Pro268Ser (SNP5) and Gly908Arg (SNP12) mutations in the *NOD2/Card15* gene

Sociodemography and clinical characteristics	Pro268Ser (SNP5)					Gly908Arg (SNP12)				
	Mutation (*n*=17)		No mutation (*n*=86)		*P*†	Mutation (*n*=24)		No mutation (*n*=79)		*P*†
	n	(%)	*n*	(%)		*n*	(%)	*n*	(%)	
Age at diagnosis*					0.729					0.969
<16 years (A1) (*n*=34)	5	(29.4)	29	(33.7)		8	(33.3)	26	(32.9)	
≥17 years (A2 and A3) (*n*=69)	12	(70.6)	57	(66.3)		16	(66.7)	53	(67.1)	
Location of disease1					0.311					0.033
Ileal (L1) (*n*=47)	9	(52.9)	38	(44.2)		11	(45.8)	36	(45.6)	
Colonic (L2) (*n*=21)	1	(5.9)	20	(23.3)		1	(4.2)	20	(25.3)	
Ileo-colonic (L3) (*n*=35)	7	(41.2)	28	(32.6)		12	(50.0)	23	(29.1)	
Behavior of disease1					0.940					0.295
Non-stricturing, non-penetrating (B1) (*n*=57)	9	(52.9)	48	(55.8)		10	(41.7)	47	(59.5)	
Stricturing (B2) (*n*=25)	4	(23.5)	21	(24.4)		8	(33.3)	17	(21.5)	
Penetrating (B3) (*n*=21)	4	(23.5)	17	(19.8)		6	(25.0)	15	(19.0)	
Perianal disease					0.119					0.984
Yes (*n*=13)	0	(0.0)	13	(15.1)		3	(12.5)	10	(12.7)	
No (*n*=90)	17	(100)	73	(84.9)		21	(87.5)	69	(87.3)	
Family history of CD					0.375					0.102
Yes (*n*=22)	5	(29.4)	17	(19.8)		8	(33.3)	14	(17.7)	
No (*n*=81)	12	(70.6)	69	(80.2)		16	(66.7)	65	(82.3)	
Extraintestinal manifestation					0.491					0.905
Yes (*n*=18)	4	(23.5)	14	(16.3)		4	(16.7)	14	(17.7)	
No (*n*=85)	13	(76.5)	72	(83.7)		20	(83.3)	65	(82.3)	

* According to the Montreal Classification.[18]. † Chi-square tests were used to estimate the *P*. If the cell count was<5, the Fisher's exact test was used to estimate the *P*

association.[30] Similarly, these three common *NOD2* alleles were reported to be associated with the German CD patients, while the frameshift homozygous mutation [Leu1007FsinsC (SNP13)] was associated with CD having fistula.[9,31] A study from the United States of America has reported that CD patients with pouchitis have a higher rate of these three NOD2 mutations compared with asymptomatic CD patients, and these mutations help in predicting pouchitis and in making the appropriate decision regarding surgical intervention.[32] A strong association of Arg702Trp (SNP8), Gly908Arg (SNP12), and Leu1007FsinsC (SNP13) mutation with risk of CD has also been suggested in the UK, particularly with the ileal disease.[33] The effects of these three *NOD2* mutations on disease manifestation and the risk of surgery in a cohort of German childhood-onset CD patients have been assessed by Lacher, *et al*.[34] The results showed that Gly908Arg (SNP12) and Leu1007FsinsC (SNP13) mutations were highly associated with CD with the Leu1007FsinsC (SNP13) mutation also conferring risk for isolated ileal and need of surgery at an earlier stage of the disease in children. In Slovenia, Gly908Arg (SNP12) and Leu1007FsinsC (SNP13) mutations have been shown to be significantly associated with CD.[35]

The current study had some limitations, including the limited sample size. However, observing statistically significant associations between variants Pro268Ser (SNP5) and Gly908Arg (SNP12) with the presence of CD indicate that the study had sufficient statistical power. Another limitation to our study is the observed deviation of genotype frequencies from the HWE. The observed deviations in the total study sample and among cases are expected in case-control studies as the selected individuals do not resemble the genetic pool of the general population.[36,37] Moreover, among control subjects, only variant Pro268Ser (SNP5) deviated from HWE; whereas, variant Gly908Arg (SNP12) was in HWE. Such deviations from HWE among control subjects can be the consequence of a selected control group, which is the case in our study, as we have selected non-IBD patients visiting the same health care facility from which CD patients were recruited. Hence, our control group does not represent the gene pool of the general population, and hence, deviations from HWE are expected.[38] A previous study investigating the association between a frameshift variant in the *NOD2* gene and the presence of CD also reported a deviation of the assessed variant from HWE.[39] Nonetheless, deviations from HWE may indicate a genotyping error, population stratification, and/or increased inbreeding. A major strength of our study is the well-characterized CD patients that minimize misclassification of disease status.

CONCLUSION

This study demonstrates that CD patients in Kuwait were less likely than controls to have the heterozygous genotype

and the homozygous for the minor allele genotype of variant Pro268Ser (SNP5). In contrast, CD patients in Kuwait were more likely to carry the homozygous for the minor allele or the heterozygous genotypes of variant Gly908Arg (SNP12) compared to controls. Hence, carrying at least one copy of the minor allele of Pro268Ser (SNP5) mutation was associated with lower odds of CD, whereas carrying at least one copy of the minor allele of Gly908Arg (SNP12) mutation was associated with increased odds of CD. Considering the pivotal role of the *NOD2* gene in sensing microbial load and in the regulation of innate immunity, the target mutations in this study may cause loss of function and contribute to CD pathogenesis.

Our study provides information about the frequency of *NOD2* gene variants in CD patients and non-IBD subjects in the Arab population in Kuwait, which is an area from where such information is generally lacking, and thus offers useful insights into the underlying genetic heterogeneity of CD in the Arabian Gulf region.

Disclosure

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Conflicts of interest

There are no conflicts of interest.

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