Background: Nucleotide-binding oligomerization domain-containing two (NOD2/CARD15) gene polymorphisms are implicated in the pathogenesis of Crohn’s disease (CD).

Aim: To describe the allelic frequency of NOD2/CARD15 gene variants among Kuwaiti patients with CD and investigate potential genotype/phenotype associations.

Methods: Adult Kuwaiti citizens with an established diagnosis of CD and healthy controls were enrolled from October 2018 to May 2020. Three common NOD2/CARD15 polymorphisms (R702W, G908R, and L1007fs) and P268S and IVS8+158 polymorphisms were screened by polymerase chain reaction/restriction analysis length polymorphism (PCR/RFLP).

Results: Ninety adult Kuwaiti patients with CD and 210 healthy subjects (as controls) were recruited. P268S, IVS8+158, G908R, and R702W minor alleles were identified in 38.9%, 21.1%, 12.2%, and 4.4% of CD patients, respectively. NOD2/CARD15 polymorphisms coexisted in 35 healthy controls (16.7%) and 21 CD patients (23.3%). Individuals with either a single or multiple polymorphism were approximately two times more likely to have CD than those with no polymorphism. Patients with multiple polymorphisms had significantly more stricturing and penetrating disease.

Conclusion: NOD2/CARD15 gene polymorphisms were significantly associated with an increased risk of disease and aggressive phenotypes among the Kuwaiti CD population.

Keywords: Crohn’s, Kuwait, NOD2/CARD15, polymorphisms
INTRODUCTION

Inflammatory bowel diseases (IBDs), including Crohn's disease (CD) and ulcerative colitis (UC), are chronic idiopathic inflammatory disorders of the gastrointestinal tract. Since 1990, the highest reported prevalence of IBD has been observed in the industrialized countries of Europe (505 UC patients per 100,000 persons in Norway; 322 CD patients per 100,000 persons in Germany),[8,9] and North America (286 UC cases per 100,000 persons in the USA; 319 CD patients per 100,000 persons in Canada).[10,11] Similarly, the highest incidences of IBD are reported in European countries and North America although these values are stable or decreasing (15.4 and 23.82 per 100,000 person-years for CD in Italy and Canada, respectively; 57.9 and 23.14 per 100,000 person-years for UC in the Faroe Islands and Canada, respectively).[12] Conversely, progressively increasing incidences were noticed in Asia and the Middle East, reaching rates of 6.3 per 100,000 person-years for UC and 5 per 100,000 person-years for CD.[13] The etiology and pathogenesis of IBD are not completely understood, but several factors are involved, including genetic, epigenetic, environmental factors (nutrition, microbiota dysbiosis), and immunologic system dysregulation.[14] In addition, the heterogeneous prevalence of IBD among different ethnic groups and familial aggregation of cases suggests a hereditary predisposition.[15] Over the last decade, multiple studies have investigated susceptibility genes for CD in various populations.[8–11] The nucleotide-binding oligomerization domain-containing protein 2/caspase recruitment domain-containing protein 15 (NOD2/CARD15) gene located on chromosome 16q12 is the first described gene associated with CD.[12] It encodes the NOD2 protein, which is mainly expressed in phagocytic immune cells, dendritic cells, and monocytes.[13] Enterocytes and Paneth cells also contain the NOD2 protein, playing an important role in the intestinal innate immune response against the bacterial cell wall.[14–16] More than 30 variants of the NOD2/CARD15 gene have been identified.[17]

Some studies reported an association between three common NOD2/CARD15 gene variants (R702W, G908R, and L1007fs) as well as P268S and IVS8+158 polymorphisms, and an increased risk of developing CD.[18–21] However, published data are conflicting, as no relation between NOD2/CARD15 variants and CD was reported in studies conducted in non-European populations, and limited data are available about IBD epidemiology among Arab populations of the Middle East.[10,22,23] In Kuwait, between 1985 and 1999, UC incidence per year was 2.8 per 100,000 persons, but to date, both the prevalence and incidence of CD are increasing with a frequency similar to that observed in Western countries.[24–26] Reports have shown that the clinical course of the disease could be less aggressive in this region compared to North America and Northern Europe.[26–28] The situation in Kuwait is still not known, and there are no published data about genetic factors. The main objective of our work is to describe for the first time the allelic frequency of NOD2/CARD15 gene variants among Kuwaiti patients with CD and to investigate potential genotype/phenotype associations.

PATIENTS AND METHODS

Patients

Our study was carried out from October 2018 to May 2020 in the gastroenterology department of Al Sabah Hospital (Kuwait). All patients were enrolled according to the following inclusion criteria: adults (age ≥18 years old), Kuwaiti nationality with an established diagnosis of CD (based on standard clinical, imaging, endoscopic, histopathological criteria, and Montreal classification[29]). Patients’ relatives with IBD and patients with cancers not related to CD were excluded from the study. Controls were healthy adults, Kuwaiti nationality identified employees of medical and surgical outpatient clinics of the hospital, randomly selected, based on the absence of a personal or familial history of CD or any other autoimmune disease.

The sample size was calculated using G*Power (version 3.1.9.6; Franz Faul, Kiel University, Germany). A sample of 90 CD patients and 210 healthy controls was large enough to detect a difference of 10% in the minor allele frequency (MAF) of the R702W mutation, based on the estimated MAF in an Iranian population, at 90% power of the study, 5% alpha error. Informed signed consent was required from patients and controls. Ethical approval of the standing committee for coordination of medical research – The
Kuwait Ministry of Health, was obtained (#1155/2018). This research was conducted in accordance with the Helsinki Declaration of 1979, and subsequently revised in 2000.

Patients’ data (including age, gender, tobacco consumption, family history of CD, disease phenotypes, and Montreal classification) were collected from their medical file registry. Healthy control data (including age, gender, and tobacco consumption) were collected from the interviews.

Genetic analyses

The blood samples were collected in ethylenediaminetetraacetic acid (EDTA) tubes and sent to the Kuwait Medical Genetic Center, where DNA was extracted, and genetic analyses were performed. The DNA was extracted from whole venous blood samples using a Maxwell™ instrument and a Maxwell 16 blood DNA purification kit (Cat. AS1010, for venous blood) according to the manufacturer’s protocol (Promega Corporation, USA). Three common NOD2/CARD15 variants (R702W, G908R, and L1007fs) and P268S and IVS8+158 polymorphisms were screened by polymerase chain reaction/restriction analysis length polymorphism (PCR/RFLP). PCR primers and the size of the restriction fragments before and after digestion are given in Table 1.[27] PCR reactions were carried out using a thermal cycler (Thermo-Fisher Scientific, Waltham MA, United States). The PCR products were checked by gel electrophoresis on a 1% agarose gel, and the product was compared against a KAPA universal ladder (Kapa Biosystems). Five microliters of each DNA-amplified fragment was digested with 2 units of the relevant restriction enzyme (New England Biolabs) at 37°C for 3 h, using buffers and conditions as per the manufacturers’ instructions. The digested DNA fragments were gel-electrophoresed on 2% agarose containing ethidium bromide at 100 volts for 30–60 min, and then visualized and photographed under UV light.

Statistical analysis

All data manipulations and analyses were performed with SPSS statistical software (version 25.0; IBM Corporation, Armonk, NY, USA). Patients' age was tested for normality with the Kolmogorov Smirnov test and was not normally distributed. Therefore, Mann-Whitney U test was used to test for significance of age across study groups (HC/CD) and carrier status (Yes/ No), and Kruskal Wallis test was used across the number of polymorphisms (None/ single/ multiple). Fisher's exact test was used in the analysis of contingency tables as an alternative to the Chi-squared test, whenever more than 20% of cells had expected values less than 5. Observed genotype frequencies of different genetic variants were tested for consistency with Hardy-Weinberg equilibrium using a readymade Excel calculator by Michael H. Court (2005-2008) (available at http://www.dr-petrek.eu/documents/HWE.xls). Significant P-value denotes that the observed frequencies of the genetic variant were not consistent with Hardy-Weinberg equilibrium. The MAF was considered causal. The association between the carrier status (MAF = Yes/ No) of NOD2/CARD15 genetic variants and the disease status (CD: Yes/ No) was analyzed using binary logistic regression and presented as odds ratio (OR), 95% confidence interval, and P-value of the Wald test. A P-value less than 0.05 was considered statistically significant. All associations involving the genetic variants were corrected for multiple comparisons using the Bonferroni method. Statistical significance was set at a Bonferroni-corrected P-value of less than 0.01.

Table 2: Demographic and Clinical characteristics of the studied population

| Variables                               | CD (n=90) | HC (n=210) | P     |
|-----------------------------------------|-----------|------------|-------|
| Gender (M/F)                            | 36/54     | 81/129     | 0.816 |
| Female (%)                              | (60%)     | (61.4%)    |       |
| Age, Mean±SD (Range)                    | 31.9±10.5 (14-55) | 34.0±9.6 (18-55) | 0.103 |
| Smokers                                 | 23 (25.6%)| 76 (36.2%) | 0.073 |
| Positive history in 1st degree relatives| 32 (35.6%)| -          |       |
| Age at diagnosis                         |           |            |       |
| <17 years                               | 13 (14.4%)| -          |       |
| 17-40 years                             | 65 (72.2%)| -          |       |
| >40 years                               | 12 (13.3%)| -          |       |
| Disease location                         |           |            |       |
| Ileum                                   | 56 (62.2%)| -          |       |
| Colonic                                 | 1 (1.1%)  | -          |       |
| Ileo-colonic                            | 33 (36.7%)| -          |       |
| Upper disease                           | 0         | -          |       |
| Disease behavior                        |           |            |       |
| Non-stricturing, non-penetrating        | 56 (62.2%)| -          |       |
| Penetrating                             | 8 (8.9%)  | -          |       |
| Strictureing                            | 19 (21.1%)| -          |       |
| Perianal disease                        | 7 (7.8%)  | -          |       |
| CD-related surgery                      | 17 (18.9%)| -          |       |

HC= Healthy control; CD= Crohn's disease; M=male; F=female; N=number
Table 3: NOD2/CARD15 Genotypes and minor allele frequencies of CD and control cases

| Genetic Variant | Study Group | Genotype, n (%) | HWE (P*) | MAF (%) | Minor Allele Carrier n | OR (95% CI) | Wald test (P*) |
|-----------------|-------------|----------------|----------|---------|------------------------|------------|---------------|
|                 |             | Wild type (-/-) | Heterozygous (+/-) | Homozygous mutant (+/+)) |               |              |               |
| P286S | Control | 161 (76.7%) | 46 (21.9%) | 3 (1.4%) | 0.028 | 52 (12.4%) | 49 (23.3%) | 1 | 0.0065 |
| CD | | 55 (61.1%) | 25 (27.8%) | 10 (11.1%) | 0.014 | 45 (25.0%) | 35 (38.9%) | 2.09 (1.23-3.56) | 1 | 0.975 |
| Total | | 216 (72.0%) | 71 (24.0%) | 13 (4.0%) | 0.889 | 97 (16.2%) | 84 (28.0%) | 1 | 0.0030 |
| IVS8+158 | Control | 166 (79%) | 44 (21.0%) | 0 | 0.042 | 44 (10.5%) | 44 (21.0%) | 1 | 0.975 |
| CD | | 71 (78.9%) | 19 (21.1%) | 0 | 0.263 | 19 (10.6%) | 19 (21.1%) | 1.01 (0.55-1.85) | | 0.975 |
| Total | | 237 (79.0%) | 63 (21.0%) | 0 | 0.263 | 19 (10.6%) | 19 (21.1%) | 1.01 (0.55-1.85) | | 0.975 |
| G908R | Control | 204 (97.1%) | 6 (2.9%) | 0 | 0.614 | 6 (1.4%) | 6 (2.9%) | 1 | 0.614 |
| CD | | 79 (87.8%) | 11 (12.2%) | 0 | 0.537 | 11 (6.1%) | 11 (12.2%) | 4.73 (1.69-13.23) | 1 | 0.0030 |
| Total | | 283 (94.0%) | 17 (5.7%) | 0 | 0.834 | 17 (2.8%) | 17 (5.7%) | 1 | 0.834 |
| R702W | Control | 202 (96.2%) | 8 (3.8%) | 0 | 0.724 | 8 (1.9%) | 8 (3.8%) | 1 | 0.724 |
| CD | | 86 (95.6%) | 4 (4.4%) | 0 | 0.829 | 4 (2.2%) | 4 (4.4%) | 1.17 (0.34-4.0) | 1 | 0.797 |
| Total | | 288 (96.0%) | 12 (4.0%) | 0 | 0.778 | 12 (2.0%) | 12 (4.0%) | 1 | 0.778 |

HC=healthy controls; CD=Crohn’s disease; HWE=Hardy‑Weinberg Equilibrium; MAF=Minor Allele Frequency. *Bonferroni’s corrected P (Statistically significant if <0.0063).
**Frequencies of NOD2/CARD15 minor alleles in CD patients and healthy controls**

Both heterozygote and homozygote P268S minor allele carriers were the most frequent polymorphisms, accounting for 38.9% of CD patients (minor allele frequency, MAF = 25%) compared to 23.3% of HCs (MAF = 12.4%) [Table 3]. IVS8+158 minor allele carriers were detected in 21% of both CD patients and HCs (MAF = 10.6% and 10.5%, respectively). G908R minor allele carriers were found in 12.2% of CD patients (MAF = 6.1%) compared to 2.9% of HC (MAF = 1.4%). R702W minor allele carriers were identified in 4.4% of CD patients (MAF = 2.2%) compared to 3.8% of HC (MAF = 1.9%). All individuals with L1007fs were wild-type homozygous. The observed genotype frequencies of all NOD2/CARD15 genetic variants were consistent with Hardy-Weinberg equilibrium (i.e., insignificant P-value). Carriers of all minor alleles were not significantly associated with the occurrence of CD. However, P268S and G908R carriers showed promising odds ratios (OR = 2.09 and 4.73, respectively), which would be statistically significant with a larger sample size.

**Association between NOD2/CARD15 variants and the demographic and clinical characteristics of CD patients**

In the CD group, NOD2/CARD15 variants did not show statistically significant associations with any demographic or clinical characteristics, given that P-values were adjusted for multiple comparisons [Table 4]. NOD2/CARD15 polymorphisms coexisted in 35 healthy controls (16.7%) and 21 CD patients (23.3%). Individuals with either a single or multiple polymorphism were approximately two times more likely to have CD than those with no polymorphism. There was no substantial difference in the likelihood of CD between individuals with single or multiple NOD2/CARD15 variant polymorphism. However, CD patients with multiple polymorphisms had significantly more aggressive disease behavior \((P = 0.025)\) and were more likely to have CD-related surgery \((P = 0.065)\). Sixty-four percent of CD patients with a single polymorphism had nonstricturing and nonpenetrating disease, compared to only 33.3% in CD patients with multiple polymorphisms. In addition, 12% of CD patients with a single polymorphism underwent restrictive surgery, compared with 38.1% among CD patients with multiple polymorphisms [Table 5].

**DISCUSSION**

Pathogenesis of IBD is still unclear; genetic factors may be associated with increased susceptibility to the disease and could play a role in its pathogenesis. NOD2/CARD15 gene was the first gene identified to correlate with increased risk for developing IBD, showing ethnic and geographic differences, with lower frequency in Asian and Arab populations than in Caucasians. In this study, we reported the genetic analysis of NOD2/CARD15 gene variants (R702W, G908R, and L1007fs) in the Kuwaiti population as well as P268S and IVS8+158 polymorphisms. Previous studies found that P268S and IVS8+158 polymorphisms of the NOD2/CARD15 gene were associated with an increased risk of developing CD in Ashkenazi Jewish and Irish populations.\(^{20,27,31}\) Over the last decade, the P268S gene variant has evolved as a predisposing genetic factor for developing CD in Asian populations.\(^{18,32,33}\)

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**Table 5: Demographic and Clinical characteristics of CD patients according to the number of NOD2/CARD15 Polymorphisms**

| Variables                  | Number of NOD2/CARD15 Polymorphisms | \(P\) |
|----------------------------|-------------------------------------|-------|
|                            | None No. (%) | Single Polymorphism No. (%) | Multiple Polymorphisms No. (%) |       |
| Gender                     |              |                              |                               |       |
| Male                       | 20 (45.5%)   | 10 (40.0%)                   | 6 (28.6%)                     | 0.430 |
| Female                     | 24 (54.5%)   | 15 (60.0%)                   | 15 (71.4%)                    |       |
| Age: Mean±SD               | 31.7±11.0    | 33.4±9.7                     | 30.9±10.7                     | 0.817 |
| Age at diagnosis           |              |                              |                               |       |
| <17 years                  | 5 (11.4%)    | 3 (12.0%)                    | 5 (23.8%)                     | 0.493 |
| 17-40 years                | 31 (70.5%)   | 19 (76.0%)                   | 15 (71.4%)                    |       |
| >40 years                  | 8 (18.2%)    | 3 (12.0%)                    | 1 (4.8%)                      |       |
| Disease location:          |              |                              |                               |       |
| Ileum                      | 28 (63.6%)   | 14 (56.0%)                   | 14 (66.7%)                    | 0.876 |
| Colon                      | 2 (4.8%)     | 0                             | 0                             |       |
| Ileo-colonic               | 15 (34.1%)   | 11 (44.0%)                   | 7 (33.3%)                     |       |
| Disease behavior:          |              |                              |                               |       |
| Non strictureing, non-penetrating | 33 (75.0%) | 16 (64.0%)                    | 7 (33.3%)                     | 0.025*|
| Penetrating                | 1 (2.3%)     | 2 (8.0%)                     | 5 (23.8%)                     |       |
| Strictureing               | 7 (15.9%)    | 5 (20.0%)                    | 7 (33.3%)                     |       |
| Perianal disease           | 3 (6.8%)     | 2 (8.0%)                     | 2 (9.5%)                      |       |
| IBD- related surgery       | 6 (14.0%)    | 3 (12.0%)                    | 8 (38.1%)                     | 0.065 |
| Family History             | 14 (31.8%)   | 8 (32.0%)                    | 10 (47.6%)                    | 0.419 |
| Smokers                    | 10 (22.7%)   | 6 (24.0%)                    | 7 (33.3%)                     | 0.634 |

CD = Crohn’s disease; M=male; F=female; No. = number. *Statistically significant \(P (<0.05)\)
In our cohort of Kuwaiti population, P268S minor allele carriers accounted for 38.9% of CD patients and 23.3% of the healthy controls, which is lower than what was recently reported in a Turkish cohort. In addition, the P268S minor allele was significantly expressed in CD patients compared to healthy controls (25% vs 12.4%). Moreover, CD patients had about two times greater odds for being P268S minor allele carriers than healthy controls (OR = 2.09, 95% CI: 1.23–3.56); however, the P-value failed to fulfill the statistical significance set at less than 0.006 to adjust for multiple comparisons. Our results reflect that the P268S polymorphism could be implicated in the susceptibility of Kuwaitis to CD, which was similarly observed in the Ashkenazi Jew, Irish, and Chinese populations.

In this study, G908R mutant genotype carriers were more frequent among CD patients in comparison to healthy controls, accounting for 12% and 3%, respectively, resulting in a higher frequency than previously reported in a Saudi cohort. Furthermore, our CD patients showed a higher frequency of G908R MAF in comparison to healthy controls (6.1% vs 1.4%), which is similar to the frequencies in Caucasians and higher than those reported in Algerian CD patients. Moreover, CD patients had about 4.7 times greater odds for being G908R minor allele carriers than healthy controls (OR = 4.73, 95% CI: 1.69 – 13.23), however, the P-value failed to fulfill the statistical significance set at less than 0.006 to adjust for multiple comparisons.

For the IVS8 gene polymorphism, no significant differences were found between CD patients and healthy controls in our study, which is consistent with previous findings in the Turkish population. Moreover, the frequency of the R702W minor allele was 2% in the healthy controls and in CD patients, which is similar to the observed frequencies in healthy Caucasians, and higher than previously reported in Arab patients with CD, but remained lower than frequencies observed in Caucasian CD patients. All individuals with the L1007f polymorphism were wild-type homozygous, which is in keeping with the finding in Bedouin Arabs and in contrast to frequencies of 6.5% and 16% previously identified in Saudi and Caucasian CD patients, respectively. In healthy Caucasian populations, the MAF of the three common NOD2/CARD15 gene variants ranged from 4% to 5% for R702W, from 1% to 2% for G908R, and from 2% to 3% for L1007E. The corresponding frequencies among Caucasian CD patients varied from 9% to 13%, from 3% to 6%, and from 7% to 16%, respectively. Familial aggregation in IBD may give consideration to sharing genetic and environmental susceptibility for several decades. In our study, no correlation was identified between disease-related surgery and NOD2/CARD15 gene mutations in CD patients, which is consistent with an earlier Turkish study finding.

Moreover, CD patients with multiple polymorphisms had a significantly more aggressive disease course (P = 0.025) and were more likely to have restrictive surgery (P = 0.065), similar to a previous study. Our study findings suggest aggressive disease behavior with a greater risk of small intestinal resection in CD patients with NOD2/CARD15 mutations, which could demonstrate the benefit of early intervention in those patient subgroups. On the other hand, no correlation was found with gender, disease location, or smoking status.

To the best of our knowledge, this is the first study to evaluate the incidence of NOD2/CARD15 polymorphisms in the Kuwait population. Furthermore, another important strength of our study is the presence of a control group, free from disease, allowing the investigation of possible correlations between genotype and disease. Finally, as expected, and in accordance with previous findings, NOD2/CARD15 minor allele carriers were more common in CD patients compared to the wild-type homozygous individuals, suggesting that these genetic alterations may be associated with increased susceptibility to the disease and play a role in its pathogenesis. However, due to sample size limitations, all genotype-related associations lost their statistical significance after correction for multiple comparisons. This hypothesis is in line with findings in Caucasian patients and could be associated with lifestyle changes in Kuwait. Social, educational, and health conditions significantly changed in the Arabian Gulf region over the last 50 years, especially in Kuwait, as evidenced by the improvement of indicators (e.g., life expectancy and infant mortality rates), higher levels of urbanization (> 85%), and per capita gross domestic product. The increasing CD incidence in Kuwait could therefore be due to not only the presence of gene polymorphisms but also to Western lifestyle changes, resulting in greater exposure to CD environmental risk factors.

**CONCLUSION**

The etiology and pathogenesis of Crohn's disease are still not clear, but interactions between environmental and immunological factors in genetically susceptible individuals may have a key role, as highlighted by several differences in incidence and prevalence among different CD populations and ethnic groups. This is the first study dealing with genetic factors in Kuwaiti patients with CD.
The NOD2/CARD15 minor alleles are more frequent in CD patients than in healthy controls. However, the frequencies of these polymorphisms are still less common among the Kuwaiti population than in Caucasians. Furthermore, larger studies might be helpful to determine whether Kuwaiti patients with CD share the same genetic profiles or have polymorphisms other than those found in Western populations.

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Conflicts of interest
There are no conflicts of interest.

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