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Frequency of Three Common Mutations of CARD15/NOD2 Gene in Jordanian Patients with Crohn's Disease

Khaled A. Jadallah[1*], Ammar K. Daoud[1], Osama N. Abu Nasra[2], Susan M. Kullab[1], Waseem T. Hamoudi[3], Niazy A. Abu Farsakh[1] and Nabil A. Bashir[4]

[1]Department of Internal Medicine, King Abdullah University Hospital, Faculty of Medicine, Jordan University of Science and Technology, Irbid, Jordan.
[2]Medical Sciences Laboratory, Faculty of Applied Medical Sciences, Jordan University of Science and Technology, Irbid, Jordan.
[3]Department of Internal Medicine, Gastroenterology and Hepatology Unit, Al-Bashir Hospital, Ministry of Health, Amman, Jordan.
[4]Department of Biochemistry and Molecular Biology, Faculty of Medicine, Jordan University of Science and Technology, Irbid, Jordan.

Authors' contributions

This work was carried out in collaboration between all authors. Authors KAJ, AKD, NAAF, and NAB designed the study. Author ONA performed the experimental work. Authors KAJ, AKD, ONAN, NAB, and WTH recruited patients, collected data and performed analysis and interpretation of results. Authors KAJ, ONA and SMK performed the literature search and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Background and Aims: CARD15/NOD2 is recognized as a major susceptibility gene for Crohn's disease. Several mutations of CARD15/NOD2 have been reported in different racial groups. We aimed to investigate the frequency of three common CARD15/NOD2 mutations in a Jordanian

**Corresponding author: Email: khaled-j@just.edu.jo;*

Crohn's disease cohort.
Methodology: Fifty one unrelated Crohn's disease patients and fifty one age- and sex-matched healthy controls were recruited at two hospitals in Jordan. Demographic and phenotypic characteristics of patients were ascertained. Allele frequencies for three CARD15/NOD2 mutations (G2722C, C2104T, 3020insC) were determined by PCR-RFLP, ARM-PCR, and direct sequencing using allele specific primers.
Results: The frequencies of G2722C alleles in Crohn's disease patients were higher but not statistically significant as compared to healthy controls (5.9% vs. 1.9%; *P* = 0.32). On the other hand, C2104T and 3020insC mutations have not been detected in Crohn's disease patients or healthy controls.
Conclusion: Our findings indicate that common mutations of CARD15/NOD2 gene in White patients with Crohn's disease are not associated with Crohn's disease in the Jordanian population. Further studies are needed to ascertain the effect of these and other mutations on Crohn's disease susceptibility and behavior in our population.

Keywords: CARD15/NOD2; mutations; Crohn's disease; Jordan; genotype.

ABBREVIATIONS

Crohn's disease (CD), ulcerative colitis (UC), capsase activation and recruitment domain 15 (CARD15), nucleotide-binding oligomerization domain 2 (NOD2), genome-wide association study (GWAS), polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), Healthy controls (HC).

1. INTRODUCTION

Crohn's disease (CD) is a chronic relapsing inflammatory condition that can affect any part of the gastrointestinal tract. The prevalence of CD is approximately 100–150 per 100,000 individuals of European ancestry [1]. The exact prevalence of CD in the Jordanian population is unknown, but it is believed to be increasing in the past two decades, similar to racially-related groups in the Middle East and other developing countries [1,2]. CD usually involves the ileum and colon but can affect any region of the gastrointestinal tract. Broad variation is observed in CD patients in terms of presentation, location, extraintestinal manifestations, and disease behavior. It is assumed that such variation is accounted for by the diverse genetic contribution to the disease in different patients [3]. In fact clinical, epidemiological and linkage data described in the past two decades have shown that genetic factors confer susceptibility to CD and influence the phenotype of the disease [4-8].

The first CD susceptibility gene identified was the CARD15 (capsase activation and recruitment domain 15), formerly called NOD2 (nucleotide-binding oligomerization domain 2) [9]. The effect of CARD15/NOD2 in CD patients was subsequently confirmed in two linkage studies [10,11]. The contribution of the CARD15/NOD2 gene to CD has been studied in different White populations with positive associations detected in up to 50% of CD patients [5,6,12-14]. However, some discrepancies were observed in these populations with respect to the prevalence and influence of CARD15/NOD2 mutations [14-16].

Earlier data from Asian countries such as Japan and China failed to show any significant association between CD and common CARD15/NOD2 mutations in Whites [17-20]. However, a recent genome-wide association study (GWAS) in a Japanese population reported a significant association with three known mutations and identified two new susceptibility loci for CD [21]. On the other hand, studies investigating the effect of different CARD15/NOD2 mutations in Mid Eastern and North African Arabs yielded conflicting results [22-25]. This is the first study that we are aware of to report the frequency of the G2722C, C2104T, and 3020insC mutations of the CARD15/NOD2 gene in a Jordanian population with CD.

2. METHODOLOGY

2.1. Study Subjects

Between September 2011 and April 2012, fifty one adult Jordanian unrelated cases of CD were recruited at King Abdullah University Hospital (Northern Jordan), and Al-Bashir Hospital (Middle Jordan). The diagnosis was based on standard clinical, laboratory, radiological,

endoscopic and histopathological criteria combined [26]. In addition, confounding conditions such as intestinal tuberculosis (TB) and amebic colitis (both common in Jordan) were ruled out with appropriate testing when there was a high index of suspicion for these diseases. In particular, patients with suspected intestinal TB (e.g. patients referred from Jordanian regions with high incidence/prevalence of TB and/or patients with respiratory or constitutional symptoms), underwent a thorough evaluation for TB infection at the time of first presentation. Clinical evaluation included a thorough history and physical examination, a chest radiograph and/or high resolution chest CT in high risk patients, and a tuberculin skin test and/or a PCR-based test whenever indicated. Additionally, a Quanti-FERON-TB Gold test (Cellestis Carnegie, Australia) was ordered for patients with suspected latent TB (typically, the test is done to patients expected to receive anti-tumor necrosis factor drugs (e.g. infliximab), to detect latent TB and treat appropriately). Finally, all biopsy specimens submitted for histopathology were examined routinely by our pathologists for the presence of acid-fast bacilli or caseating granulomas.

Patient who refused to participate and those with incomplete medical records were excluded. Out of eighty seven potential candidates evaluated, fifty one patients were ultimately enrolled in this study. The control group consisted of fifty one age- and sex-matched healthy blood donors at blood banks in the aforementioned hospitals. Informed written consent was obtained from each participant, and the study was reviewed and approved by the institutional review board at both hospitals. Of note, this work was based on the thesis presented by one of the authors (ONA) for his Master Degree in Biotechnology.

The demographic and clinical characteristics of patients were collected using an interviewer-administered questionnaire. This questionnaire included: age, gender, family history, age at onset or at diagnosis, smoking status, disease location, disease severity or behavior, disease duration, and presence of extraintestinal manifestations. Additionally, patients' medical records were thoroughly reviewed for confirmation of data gathered. In the present study, we used the Montreal classification to classify the patients in different clinical categories [27]. This classification considers three clinical variables: 1) Age at onset (A1:16 years or younger, A2: 17-40 years, A3: Over 40 years); 2) Location (L1: Terminal ileum, L2: Colon, L3: Ileocolon, L4: Upper gastrointestinal; 3) Behavior (B1: Nonstricturing, nonpenetrating, B2: Stricturing, B3: Penetrating, P: Perianal disease modifier, added in case of associated perianal disease).

2.2. DNA Extraction

Three ml of venous blood were collected in EDTA-containing tubes from each study subject and were kept at 4˚C for no more than one week before DNA extraction. The samples were collected four times weekly in the outpatient clinics and blood banks at the two participating hospitals (two days/week for each hospital). DNA was extracted using a commercially available extraction kit, the QIAamp DNA Blood Midi Kit (Qiagen, Inc., Valencia, CA, USA). Quantity and quality assays were performed by following the manufacturer's instructions. DNA samples were successfully isolated in 100% of study subjects, and CARD15/NOD2 data were available for all cases and healthy controls (HC).

2.3 Genotyping

The genotyping was carried out at Princess Haya Center for Biomedical Technologies, King Abdullah University Hospital. Samples from cases and HC were plated for genotyping, and laboratory personnel were blinded as to clinical status of the subjects.

Three types of polymerase chain reaction (PCR) were used for genotyping and detection of three mutations; the first is C2104T in exon 4, the second is G2722C in exon 8, and the third is 3020 INS C in exon 11. PCR-Restriction fragment length polymorphism (RFLP) was used to detect the first mutation, ARM-PCR to detect the second, and direct sequencing to detect the third. For RFLP-PCR to detect G2722C, the PCR was carried out in a total volume of 25µl containing 12.5µl PCR master mix (Promega, USA), 7.5µl nuclease free water (Promega, USA), 2µl of each forward 5-CCCAGCTCCTCCCTCTTC-3 and reverse primer 5-AAGTCTGTAATGTAAAGCCAC-3, and 1µl of genomic DNA. The PCR product was digested with the restriction enzymes *HhaI*. The digested samples were separated with 2% agarose gel and visualized under UV light. ARMS-PCR was used for genotyping of C2104T; two forward primers 5-ATCTGAGAAGGCCCTGCTTC-3 and 5-ATCTGAGAAGGCCCTGCTTT-3 were paired

with the same reverse primer 5-CCCACACTTAGCCTTGATG-3 in different PCR reaction (producing the same bp size) reverse primer. The genotype of a known mutation can be determined by analysis of the amplification products using agarose electrophoresis: for homozygote genotype, PCR products (either wild type or mutant type) generate a band in only one reaction. For a heterozygote genotype, PCR products generate a band in both reactions (one band in wild type reaction and one band on mutant reaction). Direct PCR product sequencing was used to detect 3020insC. The PCR reaction was carried out in a total volume of 25µl containing 12.5µl PCR master mix (Promega, USA), 7.5µl nuclease free water (Promega, USA), 2µl of each forward CTCACCATTGTATCTTCTTTTC and reverse primer GAATGTCAGAATCAGAAGGG, and 1µl of genomic DNA. PCR products purification was carried out by using Qiaquick PCR purification kit (Qiaquick, Germany). Purified PCR products were sequenced using the Big Dye Terminator Cycle Sequencing Kit version 3.1 (Qiaquick, Germany). The sequencing was: 25 cycles of 96^{o}C for 10 seconds, followed by 50^{o}C for 5 seconds and 60^{o}C for 4 minutes. The PCR sequencing products cleaning were carried out by using Centri-SEP columns purification kit (Promega, USA). The samples were then run on an ABI 310 DNA sequencer (Applied Biosystems). Sequencing results were analyzed using chromasPro software version 1.34 [28]. Reference sequences of each exon of CARD15/NOD2 (Ensembl ID: ENSG00000167207) were obtained from Ensembl Genome Browser [29] and aligned with sequencing electropherograms. Numbers and codons of amino acid residues in NOD2 gene transcript are available online [29].

2.4 Statistical Analysis

Descriptive statistics were computed by using the Statistical Package for Social Sciences (SPSS V 16.0). Fisher extract test was used to evaluate the genotype distribution and allele frequencies of the G2722C polymorphism. A *P*-value of < 0.05 was considered statistically significant.

3. RESULTS

The present study is based on data of Jordanian patients with CD from two tertiary care hospitals. A total of fifty one unrelated CD patients were genotyped for CARD15/NOD2 mutations. Fifty one healthy blood donors from the same geographical areas were used as a control group. The demographic data and clinical characteristics of our study cases are shown in Table 1.

Table 1. Demographic and clinical characteristics of Crohn's disease patients

Total number	51
Gender (Male/Female)	26/25
Age	
Range	25-67
Mean ± SD	37± 8.4
Smoking status [n (%)]	
Current smokers	7 (13.7)
Ex-smokers	11 (21.6)
Positive family history [n (%)]	3 (5.9)
Disease duration (months)	
Range	3-74
Mean ± SD	36 ± 6.7
Age at onset [n (%)]	
> 16 and < 40 years (A2)	26 (51)
≥ 40 years (A3)	25 (49)
Disease behavior [n (%)]	
Nonstricturing/ nonpenetrating (B1)	44 (86.3)
Stricturing (B2)	6 (11.7)
Penetrating (B3)	1 (2)
Perianal disease	3 (5.8)
Disease anatomical location [n (%)]	
Terminal ileum (L1)	21 (41.2)
Colon (L2)	18 (35.3)
Ileocolon (L3)	11 (21.5)
Gatsroduodenal (L4)	1 (2)
Extraintestinal manifestations [n (%)]	19 (37.3)

With regard to cigarette smoking status, eighteen out of our 51 patients (35%) were current smokers or ex-smokers. Of note, four out of the six patients with stricturing disease belonged to this category. On the other hand, only three (5.9%) patients had a positive family history for CD.

The most prevalent anatomical locations of CD in our cohort were the terminal ileum, the ileo-colon, and the colon with 41.2%, 35.3%, and 21.5%, respectively. Concerning disease behavior, only a minority of our patients (13.7%) had a complicated disease course, with stricturing disease affecting six out of fifty one patients (four in the terminal ileum and two in the right colon), and one patient with abscess formation due to perforation of the terminal ileum. Of note, more than one third of our patients had extraintestinal manifestations, with the skin and joints most commonly involved.

Healthy controls had homozygous GG genotype as shown in Fig. 1, lane 5.

Three out of the fifty one patients investigated (5.9%) had the G2722C mutation, as illustrated in Fig. 1: one as homozygous genotype GG as indicated in lane 4, one as homozygous genotype CC as indicated in lanes 7 and 8, and one as heterozygous genotype GC as in lane 6.

The C2104T mutation analysis showed that the common C allele frequencies were 100% in both CD patients and controls. There was no patient with the TT or the CT genotype (Fig. 2 and panels A, B, and C in Fig. 3).

The 3020insC polymorphism was genotyped by the sequence-PCR method. There was no patient with the 3020insC mutation, as CC genotype was 100% and 0% for genotypes +C+C or +CC (Fig. 4).

4. DISCUSSION

In the present study we investigated the frequency of three common mutations of the CARD15/NOD2 gene in adult Jordanian individuals with CD. The two participating hospitals, King Abdullah University Hospital and Al Bashir Hospital, are located in northern and middle of Jordan, respectively. These tertiary care hospitals serve almost 70% of the Jordanian population living in the two largest cities in the country, in addition to patients referred from all over the other regions. Therefore, we believe that our sample of fifty one patients is representative of the Jordanian population, which is supposed to be genetically homogeneous. The control group consisted of fifty one healthy blood donors whom we assume from the same geographical areas as the cases. Actually, in Jordan blood is ordinarily donated on an exchange basis, where relatives or friends of hospitalized patients in need for blood transfusion have to donate blood in order to allow the needed transfusion.

Interestingly, only three out of the fifty one patients (5.9%) had affected first degree family members, whereas in Whites of North America and Europe, the familial occurrence of CD has been reported to be in the range of 10 to 25% [30,31].The rarity of familial clustering of Jordanian CD patients may imply that genetic influence plays a smaller role in this racial group.

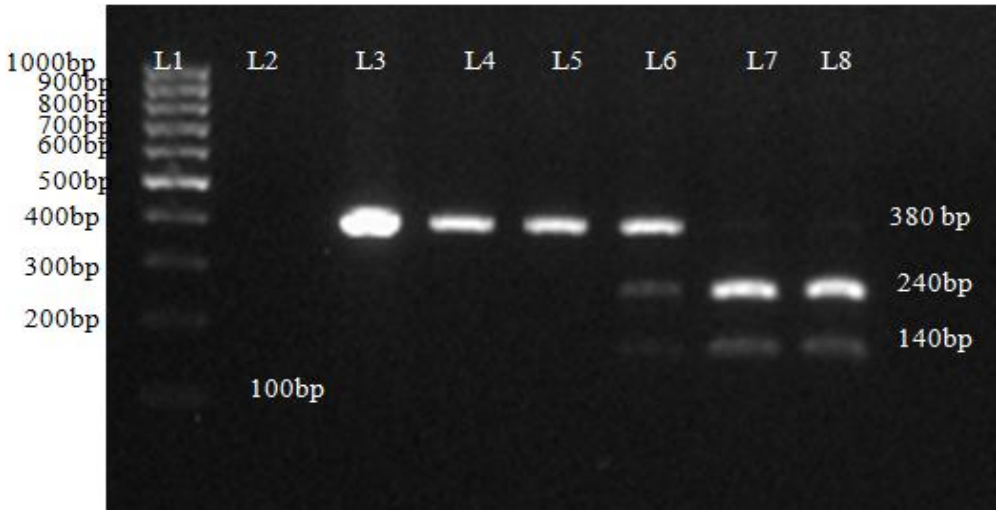

Fig. 1. Gel electrophoresis of G2722C polymorphism on exon 8. L1 is a 100 bp DNA ladder, L2 represents negative control, L3 represents the PCR product for a patient sample, and L4 is the digested product of the same patient. L6-L8 is the digested product of three PCR samples from different patients. L4 and L5 are the wild homozygous GG genotype (380bp). L6 represents the heterozygous GC genotype (380bp, 240bp and 140bp). L7 and L8 represent the mutant homozygous CC genotype (240bp and 140bp)

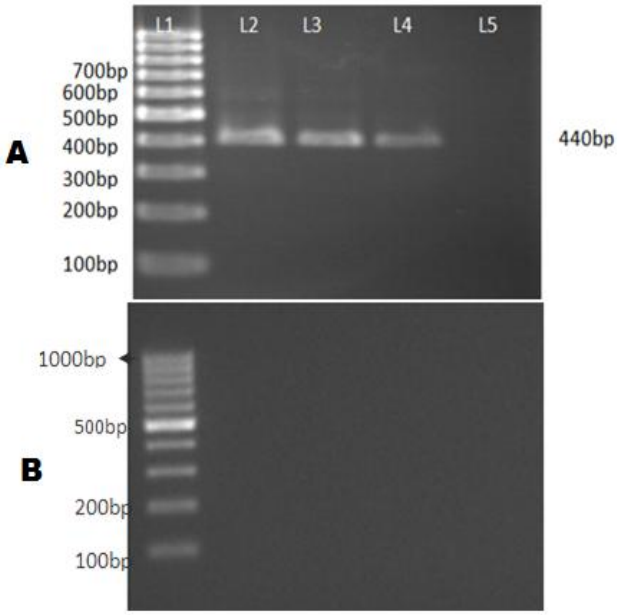

Fig. 2. Gel electrophoresis of ARM-PCR product: A shows the +2107 wild C PCR products, B represents the mutant T allele, L5 represents negative control, and L1 represents 100 bp DNA ladder. In any given numbered lane the product in the two pictures (A, B) are from the same patient.L2-L4 represent patients with wild type CC allele

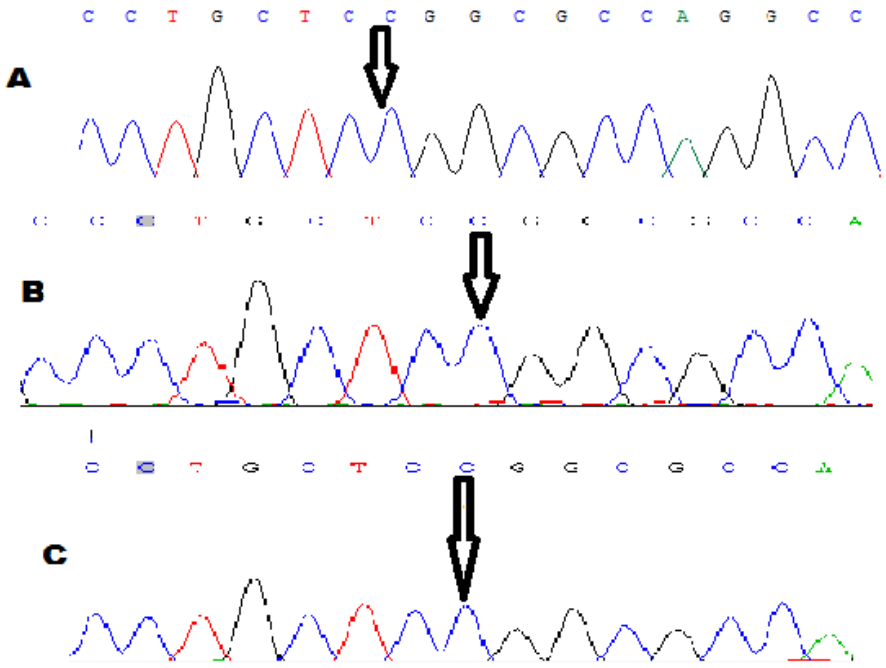

Fig. 3. Analysis of sequencing electropherograms from exon 4. This figure is a result of the confirmation study of ARM-PCR for C2104T mutation. A represents healthy control, whereas B and C represent two different patients. These electropherograms show normal sequence (arrows)

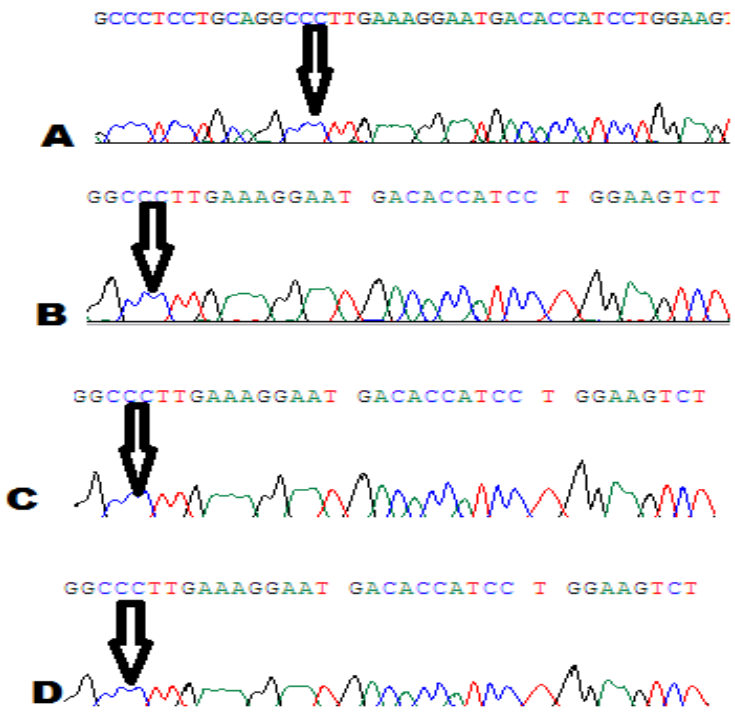

Fig. 4. Analysis of sequencing electropherograms from exon 11. Arrows indicate assumed insertion mutation position (3020insC polymorphism). A represents the healthy control, whereas B, C, and D represent three different patients. These electropherograms show normal sequenc

The mean disease duration in our CD cohort was less than three years. This could have contributed to the relatively small number of patients with complicated disease behavior (7/51) among our cases, as CD is more likely to progress to a complicated phenotype after five years of the initial diagnosis [3].

The most prevalent anatomical locations of CD in our cohort were the terminal ileum, the ileo-colon, and the colon with 41.2%, 35.3%, and 21.5%, respectively. This is similar to disease distribution data from Western countries [3] and from Japan [17].

Our genotypic analysis showed a slightly higher frequency of G2722C mutation in CD patients as compared to controls, although not statistically significant (5.9% vs.1.9%; *P* = 0.32).The other two mutations investigated (C2104T, 3020insC) were not detected in either patients or controls. In previous studies of European and White American populations, the allele frequency of the first mutation investigated, the 3020insC, has been reported to be 2%–4% [10-12]. In those studies, the mutation rate was significantly higher in CD patients as compared to patients with ulcerative colitis (UC) or HC, as illustrated in Table 2.

Moreover, in a genotype/phenotype correlation study of an American cohort [4], the 3020insC and two other CARD15/NOD2 mutations (R675W, G881R) have been shown to be associated with distinct phenotypic expression of CD, namely fibrostenosing disease. This is in contrast with the results of our study, which showed that the 3020insC mutation was absent in the fifty one CD patients investigated. On the other hand, our results are in agreement with those of studies in Japanese [17] and Chinese [19] CD populations (Table 2). In their study of three mutations (C2104T, G2722C, 3020insC) commonly found in White patients with CD, Inoue et al [17] found the mutations to be absent in CD patients, UC patients, and HC as well. Yet, the authors of the latter study did not exclude the chance of other mutations of the CARD15/NOD2 gene being involved in the susceptibility of the Japanese population to CD. Akin to the results of Inoue's study, Leong et al. [19], in their case-control study of the 3020insC and two other mutations (Arg702Trp, Gly908Arg) in sixty five Chinese patients with CD, found no mutations in

either patients or controls (Table 2). Conversely, studies of Mid Eastern and North African Arabs [22-25], who are racially related, yielded inconsistent results. In one study from Tunisia, Zouiten-Mekki et al. [23] genotyped hundred thirty CD patients and ninety HC for G2722, C2104T, and 3020insC mutations. The authors reported that the frequency of those mutations is significantly lower than that observed in Whites, and they suggested that a genetic variation of CD exists in different racial groups. Another Tunisian study [24] of the 3020insC mutation rate in seventy five CD patients, twenty five UC patients, and sixty HC showed that the frequency of the mutation was significantly higher in the CD group (P = 0.0005), as compared to the UC group (P = 0.05) or to HC (Table 2). In addition, patients with the mutation in that study were more likely to have ileal involvement, stricturing and penetrating behavior, and anti-saccharomyces cerevisiae antibodies expression. This is in contrast with the results of our study which showed absence of the 3020insC mutation in the fifty one genotyped patients. We speculate that genetic variation exists even within populations of the same racial background, such as in populations from different regions in the Arab world, and environmental factors may indeed have different influence in these regions. This variation may provide an explanation of the inconsistent results of studies from the various Arab countries.

One implication of the results of the present study is that the lower genetic susceptibility in our population may account for the relatively low prevalence of CD as compared to White populations. Actually, a comparative study of Israeli Arabs and Israeli Jewish CD cohorts found a lower rate of three CARD15/NOD2 mutations (Arg702Trp, Leu1007fsinsC, Gly908Arg) in Arabs, although the two cohorts did not differ in terms of disease phenotype [22]. The authors of that study concluded that CARD15/NOD2 mutations have an important effect on CD prevalence within a specific population but not on the phenotype. Moreover, a low prevalence of CD in Israeli Arabs was also reported in previous epidemiological studies from Israel [2,32], whereas other studies reported a high prevalence of CD despite a low CARD15/NOD2 mutation rate [1,14]. This suggests the possibility of other mutations being involved in the pathogenesis of the disease or a greater influence of environmental factors in distinct racial groups.

In the last decade, several other CARD15/NOD2 mutations (R675W, G881R, R702W, G908R, L1007sinsC, C2104T, G2722C) have been found to be associated with CD in White populations [4,5,14,33-35]. Two Dutch studies reported that the mutations R702W, G908R, and L1007sinsC are associated with fibrostenosing and perforating CD behavior [36,37]. Interestingly, in one study from Saudi Arabia of these same mutations the authors reported a significant association with CD in their patients [25]. Other studies of the same mutations in Israeli Arabs and other Mid Eastern populations yielded conflicting results [22,38-41]. These differences in the mutation rate in studies of diverse populations may reflect three factors: 1) common mutations of the CARD15/NOD2 gene in some populations may in fact be absent in other populations; 2) minor variants of the CARD15/NOD2 gene or other genes may predispose some populations to CD; 3) small sample size and heterogeneous study subjects may contribute to the conflicting results of some studies.

Although this study reveals important findings, its limitations should be noted. First, the relatively small sample size may have negatively affected the statistical power to detect differences between study subjects. Second, due to the relatively limited number of study subjects recruited in our study, we did not stratify patients into clinical subgroups according to family history, smoking status, disease behavior or location. Actually, the majority of our patients (44/51; 86%) had a negative family history for CD (Table 1). Since the absence of affected family members is recognized to be associated with low genetic susceptibility [7], it is conceivable that the low number of patients with positive family history could have impinged on the results of the present study. Furthermore, only a minority of our patients had a complicated disease behavior (13.7%), with six patients affected by strictures and one by penetrating disease. Yet again, this could have biased our results, since aggressive disease behavior is recognized to be associated with stronger genetic predisposition [4,5]. In fact all of the three patients who tested positive for the G2722C mutation in our study had complicated disease behavior. Finally, due to the small number of CD patients with positive association, relationships between genotype and phenotype—including age at onset, disease location, disease severity and behavior—could not be explored in our study.

Table 2. Frequencies of 3020insC mutation in previously reported studies

Author/Year of publication	Ref.	Population studied	Study Subjects	Key Results
Hugot JP/2001	10	French	418 CD 159 UC 103 HC	0.12 in CD 0.01 in UC 0.02 in HC
Ogura Y/2001	11	American	416 CD 182 UC 287 HC	8.2% in CD vs 3% in UC (P = 0.001) vs 4% in HC (P = 0.0018)
Hampe J/2001	12	German and British	304 CD 65 UC 272 HC	18.8% heterozygous and 6.5% homozygous in CD 6.2% heterozygous and 0% homozygous in UC 8.8 heterozygous and 0% homozygous in HC
Abreu MT/2002	4	American	201 CD 175 UC	11.4% in CD vs 3.4% in UC (P = 0.004) Also, significant association with fibrostenosing CD
Inoue N/2002	17	Japanese	350 CD 272 UC 292 HC	0% in all study subjects
Leong RW/2003	19	Chinese	65 CD 63 UC 70 controls (dyspeptic patients)	0% in all study subjects
Zouiten-Mekki L/2005	23	Tunisian	130 CD 90 HC	1% in CD vs 0% in HC
Ozen SC/2005	38	Turkish	70 CD 120 UC 106 HC	2.9% heterozygous in CD vs 0% in UC and HC; 0% homozygous in all study groups
Marrakchi R/2009	24	Tunisian	75 CD 25 UC 60 HC	18.7% heterozygous in CD vs 8% in UC and 1.67% in HC 5.3% homozygous in CD vs 0% in UC and HC

CD: Crohn's disease; UC: Ulcerative colitis; HC: Healthy controls

It should be noted that in this study we did not include patients with pediatric-onset CD, which could have influenced our results. Actually, several studies have shown that genetic susceptibility may play a more important role in the pathogenesis of pediatric CD than in adult-onset CD, and therefore, pediatric-onset CD can be anticipated to have a higher frequency of gene mutations [42-44]. De Ridder et al. [43] investigated genetic variation in CARD15 and DLG5 and reported a higher rate of mutations, 3020insC in CARD15 and rs3792876 in SLC22A4/5 in patients with pediatric-onset CD as compared to patients with adult-onset CD. More recently, a study of eighty five pediatric-onset and hundred seventeen adult-onset CD German patients was carried out with the aim of comparing disease phenotype and behavior in relation to three CARD15/NOD2 mutations (R702W, G908R, 10007fs) carriage in the two age groups [44]. The authors found that pediatric patients are more likely to have a more severe disease phenotype and aggressive CD behavior. Based on the results of those studies, it is conceivable that our cohort of adult-onset CD may in fact have infrequent gene mutations as compared to the pediatric population, and, therefore, studies of pediatric-onset CD in Jordanians are warranted.

5. CONCLUSION

The presented data indicate that the common CARD15/NOD2 mutations associated with increased susceptibility to CD in Whites do not seem to predispose to the disease in the Jordanian population. Further work is needed to determine the effect of these and other mutations on CD susceptibility and behavior in our population. In recent years, GWAS have led to the identification of approximately seventy independent genetic loci involved in the pathogenesis of CD [45]. As more GWAS reveal further genetic regions, larger population-based and case-control studies of these mutations in different racial groups will be needed to provide further insight into the pathophysiology and management of CD.

ETHICAL APPROVAL

The study procedures were approved by the Jordan University of Science and Technology - Institutional Review Board and ethical committees at the participating hospitals.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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