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Co-inheritance of variants/mutations in Malaysian patients with Crohn's disease

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ABSTRACT. Crohn's disease is a chronic, relapsing inflammatory bowel disease; it affects the mucosa and deeper layers of the digestive wall. Two Crohn's disease patients who carried the JW1 variant and two patients who carried the SNP5 variant were investigated for other co-inherited polymorphisms that could influence Crohn's disease development. Based on the sequencing results, a homozygous 5'-UTR-59 G to A variant in exon 1 (SNP6) was observed in a patient who carried SNP5, while a heterozygous SNP6 variant was detected in the other patient who carried SNP5. No other associated mutations or polymorphisms were detected in the two patients who carried the JW1 variant of the *CARD15/NOD2* gene.

Key words: Crohn's disease; Malaysian; Mutations; Variants

INTRODUCTION

Crohn's disease (CD) is a major form of chronic inflammatory bowel disease, apart from ulcerative colitis (Sugimura et al., 2003). CD usually causes swelling in the intestines, and can affect any part of the gastrointestinal tract, although it tends to affect the terminal ileum and proximal colon (Russell et al., 2004). In CD, all layers of the intestine can be affected and normal healthy bowel can be found between the sections of diseased bowel (Podolsky, 1991a).

Clinical manifestations of CD are variable among individuals in the same population. Its inflammatory process is more complex than other inflammatory bowel diseases. The main feature that distinguishes it from other inflammatory bowel diseases is the extension of the infiltrate into the deeper layers of the bowel wall (Podolsky, 1991b). This process can lead to fistulizing complications after a long period of infection, since the inflammation is transmural (Russell et al., 2004).

CD commonly develops in the teenage years or between the ages of 20 to 30, but individuals aged above 60 years also have the tendency to develop this disease, which can affect their life quality. However, phenotypes, genetics, and environmental influences are different between younger and older individuals (Polito et al., 1996). Polito et al. (1996) also found that younger patients usually have a more severe manifestation of CD compared to older patients. Therefore, they usually experience a more complicated disease course and surgery is typically required to cure the disease. Furthermore, patients who are diagnosed at early ages (especially those diagnosed before they reach 20 years of age) are often associated with positive family history (Polito et al., 1996).

In general, CD is more common in northern countries (Shivananda et al., 1996). Previous population studies in Norway and the United States showed that the incidence of CD in these 2 countries is similar, which is approximately 6 to 7.1:100,000 (Moum et al., 1996). Russell et al. (2004) reported that CD affects about 1 in 250 individuals in northern European populations. In addition, the frequency of CD is also very high among the Ashkenazi Jew population (Podolsky, 2002). On the other hand, the incidence of CD is very low in Asia and Africa. Although the exact cause of CD is unknown, both genetic and environmental factors are believed to be the major causes of this disease (Chua et al., 2011a, 2012).

Genetic factors are the important factors that contribute to the development of CD. At least 1/6 of CD patients have positive family history (Polito et al., 1996). In addition, the risk of CD is increased by approximately 5 to 10% among first-degree relatives of affected patients (Bennett et al., 1991; Podolsky, 1991a). In addition, studies also show that the risk of developing CD increases 30 times when an individual has a sibling with CD (Fielding, 1986). Moreover, studies in monozygotic twins also show that the rate of CD is very high among them, at approximately 55% (Tysk et al., 1988). However, genetic factors are less of influence in older patients (Polito et al., 1996). In a previous report, Ogura et al. (2001) found that mutations in the caspase recruitment domain-containing protein 15/nucleotide-binding oligomerization domain-containing protein 2 (*CARD15/NOD2*) gene are associated with CD. In this study, we aimed to study the co-inheritance of single nucleotide polymorphisms (SNPs) with SNP5 or JW1 in our Malaysian patients, as these variations may have the potential to mediate CD development.

MATERIAL AND METHODS

Patient sample

Four CD patients who were identified as carrying the SNP5 (patients labeled as CD17

and CD51) or JW1 (patients labeled as CD47 and CD49) mutation in a previous research study were included in the present study (Chua et al., 2009). The samples were obtained with informed consent from the University of Malaya Medical Center (Ethics Approval No. 472.55). Ethnic group, age, gender, and type of mutation for the 4 patients were recorded. Phenotype, location, and behavior of the disease in each of the patients were also classified and recorded.

DNA extraction from blood and primer design

DNA was extracted from blood using a simplified conventional method as stated previously (Tan et al., 2010).

For the primer design, the sequence of the *CARD15/NOD2* gene was obtained from the National Center for Biotechnology Information (NCBI) website (GeneID: 64127). The total length of *CARD15/NOD2* gene is approximately 35 kbp (GenBank accession No. NT_010498), which are composed of 12 exons; the length of its encoding mRNA is 4485 bp (GenBank accession No. NM_022162).

Twelve pairs of primers designed to cover all the studied regions (including intron-exon boundaries) were designed using the Primer 3 software (http://frodo.wi.mit.edu). Primer specificity checks using a similar *in silico* polymerase chain reaction (PCR) approach as reported previously were also carried out to ensure that the newly designed primers were targeted to the specific DNA regions of interest (Chua et al., 2011b; Thong et al., 2011; Ng et al., 2012a,b). The primer sequences are shown in Table 1. The JW1, SNP5, R702W, and G908R mutations had been studied in our previous report (Chua et al., 2009); therefore, these particular regions were excluded in the present study.

PCR amplification and electrophoresis

PCR in this study was performed with the primers that are listed in Table 1. The PCR mix was composed of 2.5 μL 1X reaction buffer, 1.25 μL 5% DMSO, 0.5 μL 0.2 mM dNTP, 5 μL 0.5 μM forward primer, 5 μL 0.5 μM reverse primer, 0.4 μL Taq DNA polymerase (Promega, USA), 50 ng DNA sample and an appropriate amount of distilled water. A few standard programs with slight modifications as reported previously have been used to carry out all the PCR amplifications in this study (Puah et al., 2007; Teh et al., 2010; Chan et al., 2011). The PCR products were then electrophoresed on 1.5% (w/v) agarose gels and viewed under a UV transilluminator.

DNA purification and sequencing

The DNA was purified from the PCR product using the AccuPrep PCR purification kit (Bioneer Life Science Corporation, Korea), according to manufacturer instructions. Following purification, the DNA was sent to Medigene (Malaysia) for direct sequencing. Finally, the sequencing results were analyzed using Chromas 2.33 (http://www.technelysium.com.au/chromas.html) and ClustalX (http://www.clustal.org) softwares.

RESULTS

Genomic DNA was successfully extracted from the patient samples, and the PCR products and sequencing results were also successfully obtained in this study. The overall sequencing results

showed that a nucleotide substitution was observed at position nt. 1482, exon 4 in sample CD51 (thymine and cytosine) while the reverse strand had normal sequences (adenine and guanine) at the same position without any observed nucleotide mutation/substation (Figure 1). On the other hand, Figure 2 shows that the patient labeled as CD17 had a single base pair substitution (nt. 47) at the 5'-untranslated region (UTR) of exon 1. Adenine was observed in the 5'-UTR of exon 1 in the forward strand, while thymine was observed in the reverse strand.

Table 1. Primers used in this study.

Exon	Primer	Nucleotide sequence
1	F: intron	5'-GAAGGTGGGGTTGGTAGACA-3'
	R: intron	5'-CCAGAAAACAGCCCAGAAAC-3'
2	F: intron	5'-CTCGGGTTCTGCTGGGGCT-3'
	R: intron	5'-GTGACTAAGCACCCCTTTCC-3'
3	F: intron	5'-ACATTGCTCCATCAGCCTTCC-3'
	R: intron	5'-GACTGCCCTTCCCTTTCTG-3'
4(a)	F: exon	5'-TTGTCTTCCCATTCAGCTGCC-3'
	R: exon	5'-TCAGGTACAGCTCGATGCCC-3'
4(b)	F: exon	5'-GAAGTACATCCGCACCGAG-3'
	R: exon	5'-AGGTAGAACGCGGCAAAGAA-3'
4(c)	F: exon	5'-GAATTCCTTCACATCACTTTCCAGT-3'
	R: intron	5'-ACTTAGCCTTGATGGTGCTC-3'
5+6	F: intron	5'-TCCATCTATGCAGGGTTTCCTG-3'
	R: intron	5'-GGGAGATCACAGCATTAGAGAA-3'
7	F: intron	5'-CTGCCTGCCGCTGTGTTCTC-3'
	R: intron	5'-CCTAAATCCTCAAAAGTCCCAA-3'
9	F: intron	5'-GGTCTTTCCCTGCTCTGACA-3'
	R: intron	5'-GGGATCAACAGAGATTGTGA-3'
10	F: exon	5'-TGGAGGAGAACCATCTCCAG-3'
	R: intron	5'-GGAGAGCGGCCCAGATCTTA-3'
11	F: intron	5'-CTCACCATTGTATCTTCTTTTC-3'
	R: intron	5'-CCTCAAAATTCTGCCATTCC-3'
12	F: intron	5'-TGGGTTTAAAAAGTGGAGGC-3'
	R: intron	5'-CAAACTCACAGCCTGCTCAC-3'

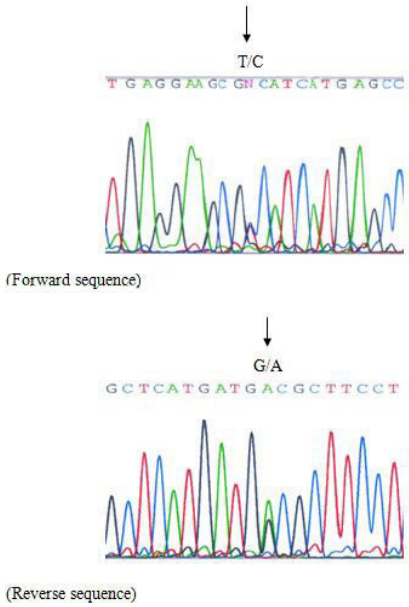

Figure 1. Partial DNA sequence of exon 4 of sample CD51.

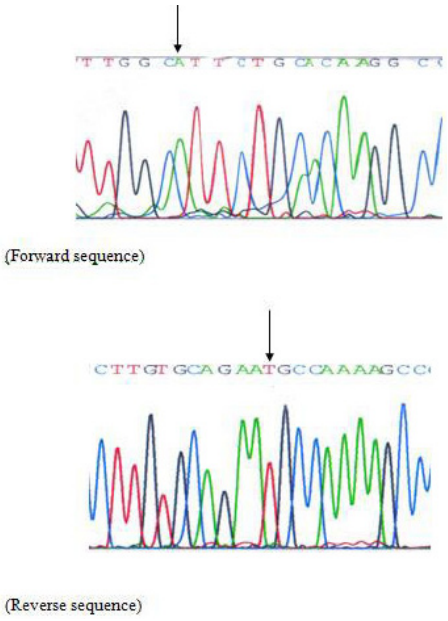

Figure 2. Partial DNA sequence of 5'-UTR in exon 1 of sample CD17.

DISCUSSION

Although the exact etiology of CD remains unknown even after numerous clinical and experimental studies have been conducted, environmental factors and genetic predisposition are confirmed to be the major factors that cause CD. In addition, a previous report revealed that the etiology of CD is associated with a dysregulated immune response to microbial organisms in the gastrointestinal tract, which subsequently leads to inflammatory cell damage (Vermeire et al., 2002).

In terms of genetic factors, *CARD15/NOD2*, a gene located at the pericentromemic region on chromosome 16, confers susceptibility to CD (Hugot et al., 2001; Ogura et al., 2001). The CARD15/NOD2 protein functions as a sensor for bacterial lipopolysaccharide through a leucine-rich repeat domain, which results in the activation of the nuclear factor-κB (NF-κB) pathway and in apoptosis under normal conditions (Vermeire et al., 2002).

Three main disease-causing mutations, R702W (exon 4, labeled as SNP8), G908R (exon 8, labeled as SNP12), and 1007 frame shift (exon 11, labeled as SNP13) were identified by Hugot et al. (2001). In our previous molecular study involving these 3 disease-causing mutations, none of our Malaysian patients with CD carried any of these mutations (Chua et al., 2009). This is likely due to genetic heterogeneity with regard to the causative factors for CD between Caucasian and Asian populations. On the other hand, the other 2 variants, SNP5 (exon 4) and JW1 (exon 8), were detected in our patients with CD. These 2 variants are commonly found in Jewish populations (Sugimura et al., 2003) and have been reported to confer susceptibility to CD in combination with other variants.

In this study, further investigation through sequencing was performed for the 4 selected patients to determine whether there is any additional disease-predisposing mutation or

variant in the *CARD15/NOD2* gene that acts in combination of SNP5 or JW1 to result in a more serious pathologic CD manifestation.

The DNA sequence chromatogram (Figure 1) shows that sample CD51 is a heterozygote variant with a single base pair substitution at nucleotide 1482 (with a base changes from C (wild type) to T (variant)) in exon 4. SNP6 (rs2066834) is a synonymous variant, as it has no changes in amino acids; the amino acid it codes for, arginine, remains the same in this case. R459R (also known as SNP6 by Hugot et al. (2001)) is located at the nucleotide binding domain of the *CARD15/NOD2* gene. The SNP6 variant, a silent mutation, was found to be in linkage disequilibrium with the 3 main mutations (R702W, G908R, and 1007fs) (Lesage et al., 2002). Of note, some studies reported that the SNP6 variant could be found in both Caucasian and Jewish populations (Lesage et al., 2002; Sugimura et al., 2003).

According to the sequencing results shown in Figure 2, the substitution of G to A was observed in sample CD17; this observation indicates that this is a homozygote variant. The variant is located at nucleotide 59 from the transcription start site in the 5'-UTR of exon 1. The 5'UTR-59 (rs2076752) is considered to be a SNP and was also reported to exist among other populations, including those from Quebec (Vermeire et al., 2002) and Jewish populations (Sugimura et al., 2003).

There are approximately 65 variants in the *CARD15/NOD2* gene (including the 1007fs frameshift mutation) that are usually found to be associated with CD in Western populations; however, these were absent in our 4 patients in this study (Lesage et al., 2002; Sugimura et al., 2003; Vermeire et al., 2004). No additional mutation or polymorphism, with the exceptions of SNP6 and 5'UTR-59, was observed from exon 1 to 12 (including intron-exon boundaries) in the *CARD15/NOD2* gene of these patients.

In conclusion, patient CD17 had a SNP5 variant and a 5'UTR-59 G to A substitution, while patient CD51 had SNP5 and SNP6 variants. No other mutation or polymorphism was detected in the 2 patients who carried the JW1 *CARD15/NOD2* gene variant. Overall, the co-inheritance of SNPs has the potential to influence disease severity. However, this study demonstrated that Malaysian patients with CD do not exhibit a similar pattern of co-inheritance of disease-predisposing SNPs.

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