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NOD2/*CARD15* variants in Malaysian patients with sporadic colorectal cancer

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ABSTRACT. Colorectal cancer (CRC) is one of the most common types of cancer in both developed and developing countries. This disease is triggered by and progresses via the sequential accumulation of multiple genetic alterations. In addition, the interaction between low-penetrance genes and environmental factors can also increase the risk of developing CRC. Since inflammatory bowel diseases (IBDs) are one of the predisposing factors for CRC, IBD-related genes might, to a certain extent, be associated with cancer initiation. The nucleotide oligomerization domain 2/caspase activating recruitment domain 15 gene (*NOD2*/*CARD15*) is the most well established gene to be associated with increased susceptibility to Crohn's disease. Thus, various studies have been performed to investigate the potential contribution of this

gene to CRC risk. In this study, we aimed to determine the frequency of the Arg702Trp, Gly908Arg, 3020insC, Pro268Ser, and JW1 variants of *NOD2*/*CARD15*, and to investigate their association with CRC susceptibility. A total of 130 CRC patients and 212 healthy controls were recruited for this study. Subsequently, real-time polymerase chain reaction (PCR) with TaqMan was performed for the genotyping of these *NOD2*/*CARD15* variants. None of the *NOD2*/*CARD15* variants was statistically associated to CRC susceptibility in our Malaysian population. Our findings were remarkably similar to those of other Asian cohorts, which indicated that these *NOD2*/*CARD15* variants exhibit genetic heterogeneity between Caucasian and Asian populations.

Key words: *NOD2*; *CARD15*; Malaysian; Colorectal cancer

INTRODUCTION

Colorectal cancer (CRC) is the third most common human malignancy and is reported as one of the leading causes for cancer mortality worldwide. In Malaysia, CRC is ranked as the second most frequent cancer and accounts for 11.4% of all cancer cases (Ferlay et al., 2010). The etiopathogenesis of sporadic CRC is grounded on multiple gene-gene and gene-environment interactions within the classical adenoma-carcinoma model (Houlston and Peto, 2004). In addition, sporadic CRC may also arise in the background of chronic inflammation as evidenced by various epidemiological and functional studies. The most prominent example is the close association between inflammatory bowel diseases (IBDs), i.e., Crohn's disease (CD) and ulcerative colitis (UC), and CRC. It was demonstrated that patients with complicating and long-standing IBDs are at an increased risk of developing CRC. Although colitis-associated CRC only contributes to 1-2% of the total CRC burden, it is considered as a serious sequela for IBDs as it accounts for 1 in 6 of all deaths among IBD patients. CRC was found to arise in up to 15% of all IBD patients throughout their lifetime (Eaden et al., 2001; Itzkowitz and Yio, 2004).

In the last few decades, the involvement of inflammation in the development of human gastrointestinal malignancies has been well studied, especially with respect to IBDs and CRC, as well as *Helicobacter pylori*-induced chronic gastritis and gastric cancer (Shacter and Weitzman, 2002). The fact that chronic inflammation predisposes a patient to CRC was supported by the presence of inflammatory histological features in the precursor lesions of CRC. It was postulated that the inflammation triggers tumorigenesis by stimulating angiogenesis, thereby inducing DNA damage and stimulating cell proliferation (Sipos et al., 2005). Thus, the inflammatory response genes that underlie IBDs, e.g., *DLG5*, *IL-4*, *OCTN*, *TNF*α, and *NOD2*, were hypothesized to be implicated in the progression of CRC (Negoro et al., 1999; Peltekova et al., 2004; Stoll et al., 2004).

Among the IBD-related genes, the genetic variants of nucleotide oligomerization domain 2/caspase activating recruitment domain 15 (*NOD2*/*CARD15*) are the most extensively studied with respect to their association to CRC susceptibility, notwithstanding various conflicting findings among different populations (Alhopuro et al., 2004; Kurzawski et al., 2004; Papaconstantinou et al., 2005; Roberts et al., 2006; Lakatos et al., 2007; Szeliga

et al., 2008). Therefore, in this study, we aimed to investigate the frequency of five genetic variants of *NOD2*/*CARD15* and their potential association to CRC susceptibility in Malaysian patients. The following variants were included in our study: two missense substitutions (Arg702Trp (rs2066844) and Gly908Arg (rs2066845)), one frameshift mutation (3020insC (rs2066847)), one background polymorphism (Pro268Ser (rs2066842)), and a novel variant, JW1 (IVS8 +158 C>T), which was recently identified among the Ashkenazi Jewish population (Sugimura et al., 2003).

MATERIAL AND METHODS

Sample cohort

A total of 130 patients and 212 control subjects were recruited for this study. The patients were all newly diagnosed with sporadic CRC and were admitted to the University Malaya Medical Center (UMMC) in Kuala Lumpur or the Queen Elizabeth Hospital in Sabah, Malaysia. The studied cases ranged in age from 40-90 years and manifested with different stages of cancer progression, ranging from tumor node metastasis (TNM) stages I-IV. On the other hand, the control samples were obtained from age-matched healthy volunteers. The blood sample collection was conducted with written informed consent, and the study was approved by the Medical Ethics Committee Board (ref no: 654.1).

Genotyping of the *NOD2/CARD15* variants

A conventional DNA extraction method (Puah et al., 2007; Chua et al., 2009b; Chua et al. 2011b) was used to isolate the genomic DNA from whole blood samples. All samples were then screened for the Arg702Trp, Gly908Arg, 3020insC, Pro268Ser, and JW1 variants by using the TaqMan SNP Genotyping Assay (Applied Biosystems; USA) as shown in Table 1. The genotyping procedure was conducted in the Applied Biosystems 7500 Fast Real-Time PCR system using the universal reaction mixture and thermal cycling conditions with initial holding step at 95°C for 20 s, followed by 40 cycles of denaturation at 95°C for 3 s, and an annealing/extension step at 60°C for 30 s as recommended by the manufacturer (Applied Biosystems).

Table 1. Pre-designed and custom TaqMan SNP genotyping assays for the screening of *NOD2*/*CARD15* variants.

NOD2/*CARD15* Variant	Nucleotide substitution	Assay ID/Primer and probe sequences
Pre-designed TaqMan SNP genotyping assay		
Arg702Trp	C>T	C_11717468_20 [(V): Allele C; (F): Allele T]
Gly908Arg	G>C	C_11717466_20 [(V): Allele C; (F): Allele G]
Pro268Ser	C>T	C_11717470_20 [(V): Allele C; (F): Allele T]
Custom TaqMan SNP genotyping assay		
JW1	C>T	Primer (F): 5'-TGG AGT AAG GAA AAA AGA CCA TTG GAT T-3' Primer (R): 5'-GAG GAC AAG GGA CAT TTC CAA GT-3' VIC-Probe: 5'-CAG AAA GAC TCG AGT GTC-3' 6-FAM-Probe: 5'-CAG AAA GAC TCA AGT GTC-3'
3020insC	Wild-type/insC	Primer (F): 5'-GTC CAA TAA CTG CAT CAC CTA CCT-3' Primer (R): 5'-ACT TCC AGG ATG GTG TCA TTC C-3' VIC-Probe: 5'-CCT GCA GGC CCT TG-3' 6-FAM-Probe: 5'-CTG CAG GCC CCT TG-3'

*(V) = VIC-Probe, (F) = 6-FAM-Probe.

Statistical analysis

Following real-time polymerase chain reaction (PCR), the frequency of each *NOD2*/*CARD15* variant was calculated by analyzing the genotyping data via TaqMan Genotyper software ver. 1.0.1 (Applied Biosystems). Similar statistical analyses, chi-squared (χ^2) test and the odds ratio (OR) with 95% confidence interval (CI) were also determined (Chua et al., 2011a).

RESULTS

Table 2 shows the distribution and frequencies of all five *NOD2*/*CARD15* variants in the CRC patient and normal control groups. No mutant for variants Arg702Trp, Gly908Arg, and 3020insC was found in our study cohort. On the other hand, the mutant T allele for both the Pro268Ser and JW1 variants was found in mutation-positive heterozygotes in our population at low frequencies of 3.5 % and 0.6 %, respectively (Table 2).

Table 2. The distribution of *NOD2*/*CARD15* variants in both CRC patients and normal controls groups.

NOD2/*CARD15* Variant	Frequency		P value	OR (95%CI)
	CRC Patients	Controls		
Arg702Trp				
C/C	130	212	-	-
C/T	0	0		
T/T	0	0		
Gly908Arg				
G/G	130	212	-	-
G/C	0	0		
C/C	0	0		
Pro268Ser				
C/C	126	204		1.2353 (0.3645 - 4.1868)
C/T	4	8	0.7334	0.8095 (0.2388 - 2.7436)
T/T	0	0		-
JW1				
C/C	129	211		0.6114 (0.0379 - 9.8601)
C/T	1	1	0.7258	1.6357 (0.1014 - 26.3791)
T/T	0	0		-
3020insC				
WT/WT	130	212	-	-
WT/insC	0	0		
insC/insC	0	0		

Through our observations, the Arg702Trp, Gly908Arg, 3020insC, Pro268Ser, and JW1 variants of the *NOD2*/*CARD15* gene were not significantly associated to CRC susceptibility in the Malaysian population ($P > 0.05$).

DISCUSSION

The *NOD2*/*CARD15* gene, which overlaps with the linkage-based IBD1 locus on chromosome 16q12, is the most prominent IBD-associated gene (Hugot et al., 1996; Ogura et al., 2001). This gene encodes a cytoplasmic protein of 1040 amino acids, which consists of two caspase recruitment domains (CARDs) at the N-terminus, a nucleotide-binding oligomerization domain, as well as eleven leucine-rich repeats (LRRs) at the C-terminus (Ogura

et al., 2001). This *NOD2*-encoded product is expressed intracellularly in peripheral blood monocytes, macrophages, granulocytes, Paneth cells, intestinal epithelial cells, etc. (Ogura et al., 2003). Physiologically, the NOD2 protein plays an important role in innate immunity by recognizing bacterial lipopolysaccharides with its LRRs, and activating the nuclear factor-kappaB (NF-κB) via its caspase recruitment domains (Ogura et al., 2001).

Based on the predicted roles of each NOD2 domain, the genetic variants of *NOD2*/*CARD15* were postulated to impair the innate immune response by either affecting the recognition of bacterial components or by dysregulating the NF-κB signaling pathway (Ogura et al., 2001). Owing to its role in the regulation of the immune response, apoptosis, cell cycle, and other cell division mechanisms, the NF-κB activity was found to be significantly elevated in several human malignancies, e.g., thyroid, breast, lung, and colorectal cancers (Gilmore et al., 1996; Chen et al., 2001). In fact, *NOD2*/*CARD15* variants were also associated with the development of several human malignancies, i.e., gastric and breast cancers, as well as non-Hodgkin's lymphoma (Huzarski et al., 2005; Rothman et al., 2006; Angeletti et al., 2009).

In the past decade, numerous studies have been performed aiming to establish a potential association between *NOD2*/*CARD15* variants and CRC susceptibility. However, the findings thus far are conflicting among different cohorts, i.e., Finnish, German, Greek, Hungarian, New Zealand, and Polish CRC patients (Alhopuro et al., 2004; Kurzawski et al., 2004; Papaconstantinou et al., 2005; Roberts et al., 2006; Lakatos et al., 2007; Szeliga et al., 2008). Moreover, the frequency of these variants also varies among different populations, i.e., between Europeans and Asians (Esters et al., 2004). In the current study, mutations at the three most common *NOD2*/*CARD15* variants, i.e., Arg702Trp, Gly908Arg, and 3020insC, were not found in our population. Thus, a positive significant association between these variants and CRC could not be established in Malaysian patients. It is noteworthy that these findings were consistent with those previously reported in Malaysian CD subjects (Chua et al., 2009a), as well as other published data on CRC in Asian cohorts, e.g., Japanese (Inoue et al., 2002; Yamazaki et al., 2002), Korean (Lee et al., 2005), Hong Konger (Leong et al., 2003), Han Chinese (Gao et al., 2005), and Indian (Pugazhendhi et al., 2008) populations.

In 2004, Kurzawski and colleagues demonstrated an association between the 3020insC mutation and an increased CRC risk in Polish patients aged > 50 years old (Kurzawski et al., 2004). Recently, a meta-analysis implicated the Arg702Trp, Gly908Arg, and 3020insC variants with an increased risk of developing CRC in Caucasians (Tian et al., 2010). A previous Greek study also documented the association between these three common variants and CRC susceptibility (Papaconstantinou et al., 2005). However, the German, Hungarian, and Finnish studies all failed to demonstrate the contribution of these variants in triggering CRC development (Alhopuro et al., 2004; Lakatos et al., 2007; Möckelmann et al., 2009).

Similar to another Malaysian study on *NOD2*/*CARD15* and CD, the mutation-positive homozygotes for both Pro268Ser and JW1 variants were absent in our population (Chua et al., 2009a). Although the mutation-positive heterozygotes were detected in our study, none of them were significantly associated to CRC susceptibility in the Malaysian patients. According to the studies from New Zealand and Poland, the Pro268Ser variant was not statistically associated to either the susceptibility or clinicopathological features of CRC (Roberts et al., 2006; Szeliga et al., 2008). In fact, published data on the association between Pro268Ser and JW1 variants with CRC are scarce, and thus their risk on CRC in different populations remains to be elucidated (Tian et al., 2010).

In summary, the *NOD2*/*CARD15* gene was not associated with CRC susceptibility in the Malaysian patients evaluated in this study. Comparison of numerous studies demonstrates the great genetic heterogeneity of *NOD2*/*CARD15* variants among individuals of different ethnic groups or geographical backgrounds, and hence, their contributions to CRC susceptibility also vary across different sample cohorts.

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