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ORIGINAL ARTICLE

Genetic variations of NOD2 and MD2 genes in hepatitis B virus infection

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Abstract Objectives: Nucleotide oligomerization domain 2 (NOD2) and myeloid differentiation protein 2 (MD-2) have crucial roles in the innate immune system. NOD2 is a member of the NOD-like receptor (NLR) family of pattern recognition receptors (PRRs), while MD-2 is a co-receptor for Toll-like receptor 4 (TLR4), which comprises another group of PRRs. Genetic variations in the *NOD2* and *MD-2* genes may be susceptibility factors to viral pathogens including hepatitis B virus (HBV). We investigated whether polymorphisms at *NOD2* (rs2066845 and rs2066844) or at *MD-2* (rs6472812 and rs11466004) were associated with susceptibility to HBV infection and advancement to related liver complications in a Saudi Arabian population. *Methods:* A total of 786 HBV-infected patients and 600 healthy uninfected controls were analyzed in the present study. HBV-infected patients were categorized into three groups based on the clinical stage of the infection: inactive HBV carriers, active HBV carriers, and patients with liver cirrhosis + hepatocellular carcinoma (HCC). *Results:* All four SNPs were significantly associated with susceptibility to HBV infection although none of the SNPs tested in *NOD2* and *MD-2* were significantly associated with

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persistence of HBV infection. We found that HBV-infected patients that were homozygous CC for rs2066845 in the *NOD2* gene were at a significantly increased risk of progression to HBV-related liver complications (Odds Ratio = 7.443 and $P = 0.044$). Furthermore, haplotype analysis found that the rs2066844-rs2066845 C-G and T-G haplotypes at the *NOD2* gene and four rs6472812-rs11466004 haplotypes (G-C, G-T, A-C, and A-T) at the *MD-2* gene were significantly associated with HBV infection in the affected cohort compared to those found in our control group. *Conclusion:* We found that the single nucleotide polymorphisms rs2066844 and rs2066845 at *NOD2* and rs6472812 and rs11466004 at *MD-2* were associated with susceptibility to HBV infection in a Saudi population.

1. Introduction

The hepatitis B virus (HBV) is a major causative agent of hepatitis and is a serious health concern worldwide. According to the World Health Organization (WHO), an estimated 240 million people globally have chronic hepatitis B infection (http://www.who.int/mediacentre/factsheets). HBV infection can result in liver cirrhosis, hepatic failure, and hepatocellular carcinoma, and is the cause of approximately 780,000 deaths each year globally (Lozano et al., 2012). The different clinical outcomes of HBV infection include recovery by clearing the virus, maintaining a chronic infection, and progression to advanced stages of HBV-related liver diseases. Phylogenetic analysis of HBV found the presence of nine genotypes, identified from A to I, with an intergroup divergence of >7.5% across the entire genome (Kramvis et al., 2005; Norder et al., 2004; Yu et al., 2010). Heterogeneity within HBV is reflected by distinct geographical distributions found globally as well as among different ethnic groups in the same country (Kramvis et al., 2005; Schaefer, 2005; Wei et al., 2010). Although the exact mechanism underlying progression of HBV infection is not clearly understood, both host and viral factors are known to influence viral gene expression, viral replication, clinical manifestations, and response to antiviral therapy. The mechanisms that cause liver damage and the extent of the damage in HBV infection are largely attributable to the host immune response to viral proteins (Nakamoto and Kaneko, 2003).

Pattern recognition receptors (PRRs) enable virus-infected cells to recognize pathogen (virus)-associated molecular patterns (PAMPs) and thus, trigger an immune response cascade. Nucleotide oligomerization domain 2 (*NOD2*), which is also known as *CARD15*, is a member of the NOD-like receptors (NLR) family of PRRs located on chromosome 16q21. The NOD2 protein has a typical nucleotide-binding domain, leucine rich protein structure which includes a C-terminal sensor domain that contains leucine-rich repeats (LRRs), a central nucleotide-binding oligomerization domain (NOD), and an N-terminal effector domain (CARD) (Liu et al., 2014). Recently, NOD2 was identified as a viral PRR important for host defense against several viruses including respiratory syncytial virus (RSV), influenza A, parainfluenza viruses, and human cytomegalovirus (HCMV) (Correa et al., 2012; Kapoor et al., 2014; Sabbah et al., 2009). In HCMV-infected cells, viral induction of NOD2 results in RIPK2-mediated activation of the NFκB pathway, the MAPKs p38, ERK, and JNK signaling pathways as well as activation of type I interferon (IFN) signaling pathways. Recent studies found increased NOD2 expression in hepatocytes where it is part of a combined immune response with Toll-like receptor (TLR) ligands, lipopolysaccharides (LPS), and polyI:C, to activate NFκB and MAPK (Scott et al., 2010). Body-Malapel et al. (2008) reported that NOD2 may regulate concanavalin A-induced liver injury (Body-Malapel et al., 2008). While single nucleotide polymorphisms (SNPs) at the *NOD2* gene were first associated with an increased risk of Crohn's disease (CD) (Hugot et al., 2001), later studies found evidence of association between SNPs at *NOD2* and other disorders including Blau syndrome (Miceli-Richard et al., 2001) and bipolar disorder (Oliveira et al., 2014). Following the initial finding by Kurzawski et al. (2004) that found polymorphisms at the *NOD2* gene conferred risk to colorectal cancer, several studies have since found associations between mutations at the *NOD2* gene and different types of cancer (Kurzawski et al., 2004; Liu et al., 2014).

TLRs are another group of PRRs combined with different accessory proteins, have an important role in the immune response. Myeloid differentiation protein 2 (MD-2), which is a soluble protein with a large hydrophobic pocket, is an accessory protein for TLRs and has a role in the recognition of bacterial lipopolysaccharides. In addition, the TLR4-MD-2 complex may respond to certain viral proteins, including the HIV Tat protein, and the resulting activated signaling cascade may result in immune dysregulation (Ben Haij et al., 2013). TLR4 is activated by hepatitis C (HCV) proteins (Howell et al., 2013) and reduces HBV replication in an interferon (IFN)-independent manner (Zhang and Lu, 2015). The TLR4-MD-2 complex binds to the pathogenic ligand which results in receptor dimerization. This TLR4-MD-2 heterodimeric structure recruits the adapter proteins MaI/TIRAP, MyD88, TRAM, and TRIF resulting in activation of the signaling pathways responsible for the regulation of inflammatory cytokines and type 1 IFN genes (Rathinam and Fitzgerald, 2011). Polymorphisms at the *MD-2* gene were associated with measles-specific humoral and cellular immunity (Dhiman et al., 2008). Verstraelen et al. (2009) reported that a SNP at the *MD-2* gene influenced the presence of *Gardnerella vaginalis* in patients with bacterial vaginalis (Verstraelen et al., 2009). In addition, a study in a Chinese population found that polymorphisms at the *MD-2* gene were associated with the occurrence or severity of neonatal necrotizing enterocolitis (NEC) (Zhou et al., 2015).

There is increasing evidence of the roles of PRRs, NOD2 and MD-2, in eliciting an immune response to viral pathogens. However, it is not known whether polymorphisms at these

genes are associated with the course of HBV infection in an Arab population. In the present study, we tested a total of four SNPs, rs2066845 and rs2066844 at the *NOD2* gene, and rs6472812 and rs11466004 at the *MD-2* gene for evidence of association with development of HBV infection and its progression to advanced liver diseases in HBV-infected Saudi patients.

2. Patients and methods

2.1. Patients

A total of 786 HBV-infected individuals of Saudi ethnic origin were included in this study. Patients were recruited from three centers in Riyadh, Saudi Arabia, the King Faisal Specialist Hospital and Research Center, the Riyadh Military Hospital, and the King Khalid University Hospital, for a 3-year period from August 2007 to August 2010. The control group in this study comprised 600 uninfected healthy Saudi individuals who were characterized by the absence of any known serological marker for HBV (HBsAg negative, anti-HBs negative, and anti-HBc negative). The study protocol conformed to the 1975 Declaration of Helsinki, and was approved by the institutional review boards of all three centers. All individuals provided informed consent prior to enrolling in the study, and their basic demographic data were recorded. Individuals positive for hepatitis B surface antigen (HBsAg) and negative for HbeAg were considered to have a non-replicative HBV infection, and if their liver enzymes were normal or nearly normal, they were categorized as asymptomatic inactive HBV carriers (Sherlock and Thomas, 1983). Subjects with repeated detection of HBsAg, negative for HbeAg, and positive for antibodies to HBeAg (anti-HBe) in addition to elevated serum ALT levels, were categorized as active HBV carriers. Liver cirrhosis in HBV-infected patients was diagnosed by liver biopsy using clinical, biochemical, or radiological criteria. Diagnosis of HCC secondary to a HBV infection was performed using computed tomography and/or magnetic resonance imaging of the liver according to guidelines published for the diagnosis and management of HCC (Abdo et al., 2006).

2.2. Genotyping of NOD2 and MD-2 SNPs

Genomic DNA was extracted from buffy coat isolated from peripheral blood samples of patients and controls using the Gentra Pure Gene kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Real-time PCR TaqMan allelic discrimination assays (Applied Biosystems, Foster City, CA, USA) were used to genotype the *NOD2* SNPs rs2066845 (assay ID: C_11717466_20) and rs2066844 (assay ID: C-11717468_20) as well as the *MD-2* SNPs rs6472812 (assay ID: C_28982107_10) and rs11466004 (assay ID: C_30755321_10). These assays were designed to use SNP-specific primers and two allele-specific TaqMan® minor groove binder (MGB) probes. Reactions were performed in 96-well plates in a total reaction volume of 25 μL using 20 ng of genomic DNA. The mixture contained 900 nM of each primer, 200 nM of each labeled probe, and TaqMan universal PCR master mix. Reaction plates were heated for 10 min at 95 °C followed by 40 cycles of 92 °C for 15 s and 60 °C for 1.5 min. Genotypes were determined using ABI Prism® 7500 Sequence Detection System software, according to the manufacturer's protocols (Applied Biosystems, Foster City, CA, USA).

2.3. Statistical analysis

Statistical analysis was performed using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA) and HaploView version 4.2. Genotype and allele distributions for SNPs at the *NOD2* and *MD-2* genes were determined for the different HBV patient groups and controls, and comparisons between groups were performed using the Pearson's χ^2 test. We tested for evidence of association between these SNPs and disease status under additive, dominant, and recessive genetic models, and findings were expressed in terms of the odds ratio (OR) and 95% confidence interval (95% CI). A $P \leqslant 0.05$ was considered statistically significant. SNPs were tested for Hardy–Weinberg equilibrium (HWE) using the DeFinetti program (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl).

3. Results

3.1. General characteristics of the subjects

A total of 786 HBV-infected patients and 600 healthy uninfected controls were analyzed in the present study. Baseline characteristics of the subjects are summarized in Table 1. Patients were subgrouped as inactive HBV carriers (N = 493), active HBV carriers (N = 209), and HBV-infected patients suffering from cirrhosis + HCC (N = 84). All subjects were Saudi nationals.

3.2. Allele and genotype comparisons

Four SNPs at the *NOD2* and *MD-2* genes were investigated for association with HBV infection and its related complications. These SNPs were rs2066845 and rs2066844 in the *NOD2* gene, and rs6472812 and rs11466004 in the *MD-2* gene. Genotype frequencies of all SNPs analyzed in this study were in HWE.

SNP genotype distributions of patients and controls are shown in Table 2. We found that all four SNPs tested in this study are significantly associated with HBV infection based on comparisons between HBV-infected patients and controls (Table 2). We found that the *NOD2* rs2066845 GC genotype was significantly associated with increased risk of HBV infection compared to GG carriers (OR = 5.386; 95% CI = 2.419–11.992; $P \leqslant 0.0001$). Furthermore, the rs2066845 C allele was associated with HBV infection risk in a dominant model (CC + GC versus GG) (OR = 1.598; 95% CI = 1.011–2.526; $P = 0.0432$). However, we did not find an increased association between susceptibility to HBV infection and the C allele compared to that found with the G allele. For *NOD2* rs2066844, we found that individuals homozygous with the TT genotype or heterozygous CT genotype compared to individuals with the CC genotype were significantly associated with risk of HBV infection (OR = 49.879; 95% CI = 6.714–370.567; $P \leqslant 0.0001$ and OR = 66.644; 95% CI = 39.101–113.590; $P \leqslant 0.0001$ respectively). We found that the rs2066844 T allele was associated with risk of HBV infection in both dominant (OR = 65.596; 95% CI = 39.091–

Table 1 Baseline and clinical characteristics of all subjects included in this study.

Variable	Inactive (n = 493)	Active (n = 209)	Cirrhosis + HCC (n = 84)	Control (n = 600)	P°-value
Age (yrs.)[a]	38.00 (29.00–50.00)	36.00 (27.00–44.50)	56.00 (49.00–62.75)	29.00 (24.00–36.00)	< 0.0001
Gender					
Male count (%)	340 (69%)	163 (78%)	71 (84.5%)	578 (96.3%)	< 0.0001
Female count (%)	153 (31%)	46 (22%)	13 (15.5%)	22 (3.7%)	
BMI[a]	27.97 (24.00–32.025)	27.18 (23.11–31.565)	24.88 (21.695–28.530)	NA	< 0.0001
ALT (IU/L)[b]	62.44 ± 316.94	79.95 ± 97.23	85.17 ± 118.28	NA	0.2270
Viral load log 10 (copies/ml)[a]	2.46 (1.623–3.37)	4.956 (4.35–7.22)	3.182 (1.875–4.579)	NA	< 0.0001

NA, not available; BMI, body-mass index; ALT, alanine aminotransferase.
[a] Values are expressed as median (interquartile ranges).
[b] Mean ± SD. p°: nonparametric test and one way ANOVA for continuous data, and Chi square test for categorical data.

Table 2 Genotypic distribution when healthy control group was compared to HBV infected patients.

Gene	SNPs	Genotype/allele distribution	Controls n = 600	Patients n = 786	OR (95% C.I.)	χ^2	P-value
NOD2	rs2066845	CC	22 (3.7%)	11 (1.4%)	0.393 (0.189–0.817)	6.70	0.0100
		GC	7 (1.2%)	48 (6.1%)	5.386 (2.419–11.992)	21.08	< 0.0001
		GG	571 (95.1%)	727 (92.5%)	1.000 (Ref.)	0.00	1.0000
		C	51 (4.2%)	70 (4.5%)	1.05 (0.726–1.518)	0.07	0.7960
		G	1149 (95.8%)	1502 (95.5%)	1.000 (Ref.)	0.00	1.0000
		CC + GC vs GG			1.598 (1.011–2.526)	4.09	0.0432
		CC vs GC + GG			0.373 (0.179–0.775)	7.53	0.0060
	rs2066844	TT	1 (0.2%)	24 (3.1%)	49.879 (6.714–370.567)	43.52	< 0.0001
		CT	15 (2.5%)	481 (61.2%)	66.644 (39.101–113.590)	532.06	< 0.0001
		CC	584 (97.3%)	281 (35.7%)	1.000 (Ref.)	0.00	1.0000
		T	17 (1.4%)	529 (33.7%)	35.295 (21.621–57.615)	447.05	< 0.0001
		C	1183 (98.6%)	1043 (66.3%)	1.000 (Ref.)	0.00	1.0000
		TT + CT vs. CC			65.596 (39.091–110.074)	550.05	< 0.0001
		TT vs. CT + CC			18.866 (2.545–139.856)	16.01	< 0.0001
MD-2	rs6472812	AA	70 (11.7%)	16 (2.0%)	0.099 (0.056–0.175)	86.47	< 0.0001
		AG	327 (54.5%)	302 (38.4%)	0.401 (0.319–0.503)	63.51	< 0.0001
		GG	203 (33.8%)	468 (59.6%)	1.000 (Ref.)	0.00	1.0000
		A	467 (38.9%)	334 (21.2%)	0.423 (0.358–0.501)	103.41	< 0.0001
		G	733 (61.1%)	1238 (78.8%)	1.000 (Ref.)	0.00	1.0000
		AA + AG vs. GG			0.347 (0.279–0.433)	90.05	< 0.0001
		AA vs. AG + GG			0.157 (0.090–0.274)	54.23	< 0.0001
	rs11466004	TT	0 (0.0%)	0 (0.0%)	nan	0.00	1.0000
		CT	502 (83.7%)	370 (47.1%)	0.174 (0.134–0.225)	195.28	< 0.0001
		CC	98 (16.3%)	416 (52.9%)	1.000 (Ref.)	0.00	1.0000
		T	502 (41.8%)	370 (23.5%)	0.428 (0.363–0.504)	105.65	< 0.0001
		C	698 (58.2%)	1202 (76.5%)	1.000 (Ref.)	0.00	1.0000
		TT + CT vs. CC			0.174 (0.134–0.225)	195.28	< 0.0001
		TT vs. CT + CC			nan	0.00	1.0000

Risk allele marked in bold letters.

110.074; $P < 0.0001$) and recessive models (OR = 18.866; 95% CI = 2.545–139.856; $P \leqslant 0.0001$). In addition, the T allele was associated with an increased risk of HBV infection compared to that found with the C allele (OR = 35.295; 95% CI = 21.621–57.615; $P < 0.0001$).

We found that the *MD-2* rs6472812 A allele was associated with a decreased risk of HBV infection (OR = 0.423; 95% CI = 0.358–0.501; $P < 0.0001$). Similarly, the rs6472812 A allele was significantly associated with susceptibility to HBV infection in the opposite direction for both individuals with the homozygous AA (OR = 0.099; 95% CI = 0.056–0.175; $P \leqslant 0.0001$) or heterozygous AG (OR = 0.401; 95% CI = 0.319–0.503; $P \leqslant 0.0001$) genotypes compared to individuals with the GG genotype. Furthermore, we found that the rs6472812 A allele was significantly associated with risk of HBV infection in the opposite direction under both dominant (OR = 0.347; 95% CI = 0.279–0.433; $P < 0.0001$) and recessive (OR = 0.157; 95% CI = 0.090–0.274; $P \leqslant 0.0001$) models, findings that may indicate a protective role for the A allele against development of HBV infection. For *MD-2* rs11466004, we found that the T allele was significantly associated with a decreased risk of HBV infection compared to that seen with the C allele (OR = 0.428; 95% CI = 0.363–0.504; $P < 0.0001$). Similarly, the rs11466004 T allele was significantly associated with HBV infection in the opposite direction compared to individuals with the heterozygous CT genotype (OR = 0.174; 95% CI = 0.134–0.225; $P < 0.0001$) and under a dominant model (OR = 0.174; 95% CI = 0.134–0.225;

Table 3 Genotypic distribution when inactive patients were compared to active, cirrhosis and HCC groups.

Gene	SNPs	Genotype/allele distribution	Inactive HBV patients n = 493	Active HBV + cirrhosis + HCC patients n = 293	OR (95% C.I.)	χ^2	P-value
NOD2	rs2066845	CC	7 (1.4%)	4 (1.3%)	0.939 (0.272–3.238)	0.01	0.9210
		GC	34 (6.9%)	14 (4.8%)	0.677 (0.357–1.284)	1.44	0.2290
		GG	452 (91.7%)	275 (93.9%)	1.000 (Ref.)	0.00	1.0000
		C	48 (4.9%)	22 (3.8%)	0.762 (0.455–1.276)	1.07	0.3005
		G	938 (95.1%)	564 (96.2%)	1.000 (Ref.)	0.00	1.0000
		CC + GC vs. GG			0.722 (0.406–1.281)	1.25	0.2640
		CC vs. GC + GG			0.961 (0.279–3.311)	0.00	0.9500
	rs2066844	TT	13 (2.7%)	11 (3.8%)	1.580 (0.682–3.659)	1.16	0.2820
		CT	297 (60.2%)	184 (62.8%)	1.157 (0.851–1.572)	0.87	0.3510
		CC	183 (37.1%)	98 (33.4%)	1.000 (Ref.)	0.00	1.0000
		T	323 (32.8%)	206 (35.2%)	1.113 (0.897–1.380)	0.94	0.3310
		C	663 (67.2%)	380 (64.8%)	1.000 (Ref.)	0.00	1.0000
		TT + CT vs. CC			1.175 (0.867–1.592)	1.08	0.2990
		TT vs. CT + CC			1.440 (0.637–3.258)	0.78	0.3790
MD-2	rs6472812	AA	9 (1.8%)	7 (2.4%)	1.290 (0.472–3.526)	0.25	0.6180
		AG	192 (39.0%)	110 (37.5%)	0.951 (0.704–1.283)	0.11	0.7400
		GG	292 (59.2%)	176 (60.1%)	1.000 (Ref.)	0.00	1.0000
		A	210 (21.3%)	124 (21.2%)	0.992 (0.772–1.274)	0.00	0.9485
		G	776 (78.7%)	462 (78.8%)	1.000 (Ref.)	0.00	1.0000
		AA + AG vs. GG			0.966 (0.719–1.297)	0.05	0.8167
		AA vs. AG + GG			1.316 (0.485–3.573)	0.29	0.5890
	rs11466004	TT	0 (0.0%)	0 (0.0%)	nan	0.00	1.0000
		CT	228 (46.2%)	142 (48.5%)	1.093 (0.818–1.460)	0.36	0.5472
		CC	265 (53.8%)	151 (51.5%)	1.000 (Ref.)	0.00	1.0000
		T	228 (23.1%)	142 (24.2%)	1.063 (0.836–1.352)	0.25	0.6165
		C	758 (72.9%)	444 (75.8%)	1.000 (Ref.)	0.00	1.0000
		TT + CT vs. CC			1.093 (0.818–1.460)	0.36	0.5472
		TT vs. CT + CC			nan	0.00	1.0000

Table 4 Genotypic distribution when active patients were compared to cirrhosis + HCC groups.

Gene	SNPs	Genotype/allele distribution	Active HBV patients n = 209	Cirrhosis + HCC patients n = 84	OR (95% C.I.)	χ^2	P-value
NOD2	rs2066845	CC	1 (0.5%)	3 (3.6%)	7.443 (0.763–72.637)	4.07	0.0440
		GC	12 (5.7%)	2 (2.4%)	0.414 (0.090–1.890)	1.38	0.2410
		GG	196 (93.8%)	79 (94.0%)	1.000 (Ref.)	0.00	1.0000
		C	14 (3.3%)	8 (4.8%)	1.443 (0.594–3.506)	0.66	0.4160
		G	404 (96.7%)	160 (95.2%)	1.000 (Ref.)	0.00	1.0000
		CC + GC vs. GG			0.954 (0.329–2.765)	0.01	0.9310
		CC vs. GC + GG			7.704 (0.790–75.142)	4.26	0.0390
	rs2066844	TT	8 (3.8%)	3 (3.6%)	0.810 (0.201–3.265)	0.09	0.7670
		CT	134 (64.1%)	50 (59.5%)	0.806 (0.472–1.378)	0.62	0.4310
		CC	67 (32.1%)	31 (36.9%)	1.000 (Ref.)	0.00	1.0000
		T	150 (35.9%)	56 (33.3%)	0.893 (0.612–1.304)	0.34	0.5585
		C	268 (64.1%)	112 (66.7%)	1.000 (Ref.)	0.00	1.0000
		TT + CT vs. CC			0.807 (0.475–1.370)	0.63	0.4260
		TT vs. CT + CC			0.931 (0.241–3.596)	0.01	0.9170
MD-2	rs6472812	AA	4 (1.9%)	3 (3.6%)	1.694 (0.367–7.832)	0.47	0.4950
		AG	83 (39.7%)	27 (32.1%)	0.735 (0.428–1.261)	1.26	0.2620
		GG	122 (58.4%)	54 (64.3%)	1.000 (Ref.)	0.00	1.0000
		A	91 (21.8%)	33 (19.6%)	0.878 (0.562–1.372)	0.33	0.5690
		G	327 (78.2%)	135 (80.4%)	1.000 (Ref.)	0.00	1.0000
		AA + AG vs. GG			0.779 (0.461–1.316)	0.87	0.3500
		AA vs. AG + GG			1.898 (0.416–8.669)	0.71	0.4010
	rs11466004	TT	0 (0.0%)	0 (0.0%)	nan	0.00	1.0000
		CT	94 (45.0%)	48 (57.1%)	1.631 (0.979–2.719)	3.55	0.0595
		CC	115 (55.0%)	36 (42.9%)	1.000 (Ref.)	0.00	1.0000
		T	94 (22.5%)	48 (28.6%)	1.379 (0.919–2.069)	2.42	0.1200
		C	324 (77.5%)	120 (71.4%)	1.000 (Ref.)	0.00	1.0000
		TT + CT vs. CC			1.631 (0.979–2.719)	3.55	0.0595
		TT vs. CT + CC			nan	0.00	1.0000

$P < 0.0001$). However, this finding with the rs11466004 T allele should be interpreted with caution, as the TT genotype was not found in either healthy controls or HBV-infected patients.

We next determined whether any of these four SNPs were associated with HBV infection persistence by comparing patients from the active and cirrhosis + HCC groups with inactive HBV carriers. We did not find evidence of association for any of the polymorphisms (Table 3). We also tested whether these variants from the *NOD2* and *MD-2* genes were associated with progression of HBV infection to more severe stages of related liver diseases. Only rs2066845 in the *NOD2* gene was significantly associated with the development of cirrhosis or cirrhosis + HCC among HBV infected patients compared to active HBV carriers (Table 4). In addition, we found that the rs2066845 C allele significantly increased risk of developing HBV-related liver diseases in individuals with the homozygous CC genotype compared to individuals with the GG genotype (OR = 7.443; 95% CI = 0.763–72.637; $P = 0.044$) with a similar finding observed under a recessive model (OR = 7.704; 95% CI = 0.790–75.142; $P = 0.039$).

3.3. Analysis of gene-gene interaction

The allele frequencies of both rs2066845 and rs11466004 were analyzed in the active group and compared to patients diagnosed with cirrhosis and HCC (Table 5). Our results show significant differences when GG + CG genotype of rs2066845 was combined with CC genotype of rs11466004 (OR = 1.765; 95% CI = 1.056–2.950; $P = 0.029$). Similarly, the combined frequency of GG of rs2066845 and CC of rs11466004 (OR = 1.738; 95% CI 1.036–2.915; $P = 0.035$) and the frequency of GG of rs2066845 and CT + TT of rs11466004 (OR = 0.573; 95% CI 0.344–0.954; $P = 0.032$) were significantly different between the analyzed groups (Table 5).

3.4. Haplotype association analyses

Three haplotypes were identified in the *NOD2* gene following our analysis between HBV-infected patients and healthy controls (Table 6). We found an increased frequency of the rs2066844C-rs2066845G (C-G) haplotype in controls compared to the C-G haplotype frequency seen in the patient cohort ($\chi^2 = 370.513$, $P < 0.0001$). However, we found a significantly increased frequency of the rs2066844-rs2066845 T-G haplotype in patients compared to that in the controls ($\chi^2 = 425.447$, $P < 0.0001$). Furthermore, haplotype analysis of the rs6472812 and rs11466004 polymorphisms at the *MD-2* gene identified four statistically significant haplotypes between comparisons of HBV-infected patients and healthy controls (Table 6). An increased frequency of the rs6472812G-rs11466004C (G-C) haplotype was found in patients compared to that in the controls ($\chi^2 = 169.97$, $P < 0.0001$), while in contrast, an increased frequency of the G-T haplotype was found in healthy controls compared to that in the patients ($\chi^2 = 22.023$, $P < 0.0001$). Similarly, increased frequencies of the A-C and A-T haplotypes were found in controls compared to those in the HBV-infected patients ($\chi^2 = 20.322$ and $\chi^2 = 87.076$, respectively; both $P < 0.0001$) (Table 6).

We performed similar haplotype frequency analyses between inactive carriers and the combined patient group (active carriers and cirrhosis + HCC) (Table 7). No evidence of association was found between haplotypes of SNPs at the *NOD2* gene and the *MD-2* gene and patient classification.

Table 5 Interactions between NOD2 and MD2 polymorphisms in active patients compared with patients diagnosed with cirrhosis + HCC.

NOD2 rs2066845	MD2 rs11466004	Active/cirrhosis + HCC	OR (95% CI)	P value
GG + CG	CC	114/34	1.765 (1.056–2.950)	**0.029**
GG + CG	TT + CT	94/47	0.643 (0.387–1.071)	0.089
GG	CC	108/32	1.738 (1.036–2.915)	**0.035**
CC + CG	CC	7/4.00	0.693 (0.197–2.432)	0.565
CC + CG	TT + CT	6/1.00	2.453 (0.291–20.692)	0.677
GG	CT + TT	88/47	0.573 (0.344–0.954)	**0.032**

Table 6 Haplotype frequencies between healthy control group and HBV-infected patients.

Gene	Block		Freq.	HBV patients, control ratio counts	HBV patients, control frequencies	Chi square	P-value
NOD2	rs2066844	rs2066845					
	C	G	0.766	992.0 : 580.0, 1132.0 : 68.0	0.631, 0.943	370.513	< 0.0001
	T	G	0.19	510.0 : 1062.0, 17.0 : 1183.0	0.324, 0.014	425.447	< 0.0001
	C	C	0.037	51.0 : 1521.0, 51.0 : 1149.0	0.032, 0.042	1.944	0.1632
MD-2	rs6472812	rs11466004					
	G	C	0.502	958.8 : 613.2, 432.0 : 768.0	0.610, 0.360	169.970	< 0.0001
	G	T	0.209	279.2 : 1292.8, 301.0 : 899.0	0.178, 0.251	22.023	< 0.0001
	A	C	0.184	243.2 : 1328.8, 266.0 : 934.0	0.155, 0.222	20.322	< 0.0001
	A	T	0.105	90.8 : 1481.2, 201.0 : 999.0	0.058, 0.168	87.076	< 0.0001

Table 7 Haplotype frequencies between inactive carriers versus active carriers + cirrhosis + HCC.

Gene	Block		Freq.	Active carriers + cirrhosis + HCC and inactive, ratio counts	Active carriers + cirrhosis + HCC and inactive frequencies	Chi square	P-value
NOD2	rs2066844	rs2066845					
	C	G	0.631	364.5 : 221.5, 627.2 : 358.8	0.622, 0.636	0.315	0.5745
	T	G	0.325	199.5 : 386.5, 310.8 : 675.2	0.340, 0.315	1.071	0.3008
	C	C	0.033	15.5 : 570.5, 35.8 : 950.2	0.026, 0.036	1.122	0.2895
	T	C	0.012	6.5 : 579.5, 12.2 : 973.8	0.011, 0.012	0.055	0.8151
MD-2	rs6472812	rs11466004					
	G	C	0.604	351.5 : 234.5, 598.7 : 387.3	0.600, 0.607	0.083	0.7726
	G	T	0.183	110.5 : 475.5, 177.3 : 808.7	0.189, 0.180	0.188	0.6645
	A	C	0.16	92.5 : 493.5, 159.3 : 826.7	0.158, 0.162	0.038	0.8461
	A	T	0.052	31.5 : 554.5, 50.7 : 935.3	0.054, 0.051	0.040	0.8406

Table 8 Haplotype frequencies between active carriers versus cirrhosis + HCC.

Gene	Block		Freq.	Cirrhosis + HCC, active ratio counts	Cirrhosis + HCC, active frequencies	Chi square	P-value
NOD2	rs2066844	rs2066845					
	C	G	0.622	107.4 : 60.6, 257.2 : 160.8	0.640, 0.615	0.299	0.5848
	T	G	0.34	52.6 : 115.4, 146.8 : 271.2	0.313, 0.351	0.784	0.3759
	C	C	0.026	4.6 : 163.4, 10.8 : 407.2	0.027, 0.026	0.008	0.9279
	T	C	0.011	3.4 : 164.6, 3.2 : 414.8	0.020, 0.008	1.751	0.1858
MD-2	rs6472812	rs11466004					
	G	C	0.6	94.8 : 73.2, 257.0 : 161.0	0.564, 0.615	1.287	0.2567
	G	T	0.188	40.2 : 127.8, 70.0 : 348.0	0.240, 0.168	4.070	0.0436
	A	C	0.157	25.2 : 142.8, 67.0 : 351.0	0.150, 0.160	0.092	0.7620
	A	T	0.054	7.8 : 160.2, 24.0 : 394.0	0.046, 0.057	0.293	0.5881

Also, we performed haplotype analysis for SNPs in both genes between active carriers and patients with cirrhosis + HCC. The results show a statistically significant difference in the frequency of the rs6472812-rs11466004 G-T haplotype ($\chi^2 = 4.07$, $P = 0.0436$) between the groups (Table 8). No evidence of association for other haplotype combinations was found.

4. Discussion

NOD2 is a member of evolutionarily conserved NLR family with a basic three-component structure consisting of a C-terminal sensor domain, a central nucleotide-binding oligomerization domain (NOD), and an N-terminal effector domain (CARD) (Deng and Xie, 2012). NOD2 has a role in the detection of microbial components, the regulation of apoptosis, and chronic inflammatory conditions (Girardin et al., 2003). Previous studies have found that structural modifications of the *NOD2* gene stimulate innate immunity by activating NF-κB, mitogen-activated protein kinases (MAPK), and interferon (IFN) regulatory factors (IRFs) (Kharwar et al., 2016). Further establishing its role as a viral PRR important for host defense against virus infection, the activation of NOD2 results in initiation of the mitochondrial antiviral signaling (MAVS)-IRF3-IFN pathway in respiratory syncytial virus (RSV), influenza A, and parainfluenza viral infections (Sabbah et al., 2009). Kapoor et al. (2014) reported that NOD2 had a central role in detecting viruses including persistent DNA viruses such as HCMV (Kapoor et al., 2014). The authors further proposed that NOD2 induction results in activation of both a classic signaling pathway involving RIPK2-TAK1-NF-κB and alternative pathways that may be RIPK2-independent including stimulation of type I IFN and autophagy (Kapoor et al., 2014). There is increasing evidence that innate immune responses mediated by PRRs play a crucial role in HBV pathogenesis and hepatocellular injury (Busca and Kumar, 2014). In addition to its expression in different effector cells of immune system, NOD2 is highly expressed in hepatocytes (Scott et al., 2010). Recently, several studies investigated whether there was a relationship between genetic variations in the *NOD2* gene and disease risk. A study conducted by Kanaan et al. (2012) found a significant association between three SNPs (rs5743293, rs2066844, and rs2066845) at the *NOD2* gene and inflammatory bowel disease in a Caucasian population (Kanaan et al., 2012). In addition, these polymorphisms at *NOD2* are associated with CD and impair cellular responses to bacterial peptidoglycans (Wang et al., 2014). Another study reported that rs2066844 and rs2066845 at the *NOD2* gene are associated with increased susceptibility to Guillain–Barre syndrome, which is an acute inflammatory neuropathy of the peripheral nervous system (Kharwar et al., 2016). A meta-analysis by Liu et al. (2014) found that the SNPs rs2066844, rs2066845, and rs2066847 at the *NOD2* gene are associated with an increased risk of cancer (Liu et al., 2014). In addition, a polymorphism at the *NOD2* gene was associated with increased risk of liver and intestinal failure in transplant patients (Ningappa et al., 2011). In the present study, we tested two SNPs (rs2066844 and rs2066845) at the

NOD2 gene for evidence of association with HBV. Our association analysis was performed between patient groups at different stages of HBV infection and healthy control subjects. We found that the genotype frequencies of individuals with the rs2066845 GC genotype or those having either the CC or CG genotype were significantly associated with increased risk of HBV infection compared to the genotype frequencies found in healthy controls. Both of the TT and CT genotypes in rs2066844 showed significant associations with increased risk of HBV infection. In addition, the rs2066844 T allele was associated with increased susceptibility to HBV infection under both dominant and recessive models. Similar findings with these SNPs were reported by Liu et al. (2014) to determine whether there was evidence of association of these variants at the *NOD2* gene with cancer risk (Liu et al., 2014). Although we found no evidence of association with these SNPs at the *NOD2* gene with persistent HBV infection, we found that rs2066845 was significantly associated with progression to cirrhosis and HCC in patients with HBV infection. The homozygous CC genotype of rs2066845 was significantly associated with progression to HBV-related liver diseases in the active carrier group compared to patients with cirrhosis + HCC. Interestingly, the rs2066845 CC genotype was significantly associated in the opposite direction (OR = 0.393; 95% CI = 0.189–0.817; $P = 0.01$) with susceptibility to HBV infection compared to healthy controls.

The *NOD2* gene is comprised of 12 exons and encodes a protein consisting of 1040 amino acids. The missense mutation rs2066844 is located in exon 4 and results in the substitution of arginine at position 702 with tryptophan, while rs2066845 in exon 8 leads to the substitution of glycine at position 908 with arginine (Boukercha et al., 2015). Boukercha et al. (2015) stated that these mutations affect either the structure of the carboxy-terminal LRR domain of the protein or the adjacent region (Boukercha et al., 2015). Kapoor et al. (2014) first reported that *NOD2* activation by HCMV was an important step toward restricting viral replication, and that mutations in the *NOD2* gene resulted in enhanced HCMV replication (Kapoor et al., 2014). Although the effects of *NOD2* activation on signaling pathways are pathogen-determined, our finding that increased susceptibility to HBV infection was associated with a polymorphism in the *NOD2* gene may be because of its effect on viral replication. The different effects of the rs2066845 CC genotype on susceptibility to HBV infection and progression to HCC may be attributed to two points: (1) *NOD2* activates different signaling pathways, and (2) it has diverse functions from its role as a regulatory molecule for inflammatory processes (Moreira and Zamboni, 2012) to the multifactorial etiology of HCC. A detailed investigation of the mechanisms that are affected by *NOD2* gene mutations in HBV-infected patients would be important to confirm these speculative inferences. The minor allele frequencies of rs2066845 (0.044) and rs2066844 (0.197) in the present study were consistent with data obtained from the 1000 Genomes Project (phase 3). Furthermore, the loss of heterozygosity observed in rs2066845 may be because of widespread consanguinity in the Saudi population. Similar observations were made by AlSuhaibani et al. (2015) in their study on ABO and Rh blood group polymorphisms in a Saudi population (AlSuhaibani et al., 2015).

We also found significant differences in the frequency of two haplotypes at the *NOD2* gene between HBV patients and healthy controls, but not among haplotype frequency comparisons between clinical groups. We found an increased frequency of the rs2066844-rs2066845 T-G haplotype in the patient cohort compared to the control group with a frequency of 0.324 and 0.014, respectively. This haplotype includes the minor T allele of rs2066844, confirming its association with increased susceptibility to HBV infection.

MD-2 is a protein important for ligand recognition by TLR4, and recognizes several components of pathogens and endogenous molecules which are produced during abnormal or aberrant situations, such as tissue damage (Vaure and Liu, 2014). It has been proposed that TLR4 ligands may recognize and bind to the TLR4/MD-2 complex instead of directly to TLR4 alone (Vaure and Liu, 2014). Several reports have found that MD-2 may be partially responsible for determining the binding specificity of the TLR4/MD-2 receptor complex, and thereby influences TLR4 function (Lizundia et al., 2008; Vasl et al., 2009). TLR4 recognizes many viral proteins that result in the activation of signaling pathways and subsequently, immune dysregulation (Ben Haij et al., 2013; Kurt-Jones et al., 2000; Rassa et al., 2002). Genetic variations within the *TLR4* gene are associated with susceptibility to viral infection (Awomoyi et al., 2007), bacterial infection (Taylor et al., 2012), hepatic fibrosis (Guo et al., 2009), and HCV-associated HCC (Agundez et al., 2012). In our previous study, we found that SNPs from the *TLR4* gene were significantly associated with HCV-infection among a Saudi Arabian population (Al-Qahtani et al., 2014). In the present study, we focused on testing polymorphisms at the *MD-2* gene for association with HBV infection because it was understudied. The *MD-2* gene maps to chromosome 8 and contains 5 exons. Our analysis found that the two polymorphisms at the *MD-2* gene, rs6472812 and rs11466004, were associated with susceptibility to HBV infection. We found decreased frequencies of rs6472812A and rs11466004T in patients compared to those seen in controls. These findings indicate that variations at both SNPs are significantly associated with a decreased risk of HBV infection. We propose that the rs6472812A allele and the rs11466004T allele exert potentially protective roles against the development of HBV infection. Previous studies have identified variations at the *MD-2* gene that are linked to susceptibility to diseases. Gu et al. (2007) found that polymorphisms in the promoter of the *MD-2* gene were associated with increased susceptibility to complications such as organ dysfunction and sepsis after major trauma (Gu et al., 2007). Hamann et al. (2004) investigated the functional consequences of the A103G polymorphism in the *MD-2* gene, and found reduced MD-2 activity as determined by measuring NF-kB-activation (Hamann et al., 2004). Another study found that a mutation at the *MD-2* gene resulted in decreased cellular responsiveness to bacterial endotoxin (Vasl et al., 2008).

Both SNPs at the *MD-2* gene tested in this study are located in intronic regions rendering it difficult to account for how these variations influence susceptibility to HBV-infection. Moreover, the proposed protective role of these *MD-2* polymorphisms may involve an innate immune co-receptor that is capable of generating a plethora of inflammatory responses, which can be favorable. This proposal is consistent with findings from a previous study that found a decreased risk of infection associated with SNPs at the *TLR4* gene that are usually linked to an increased risk of disease (Misch and Hawn, 2008). Experimental evidence is crucial to fully understand

the functional significance of the polymorphisms tested at the *MD-2* gene. Furthermore, we found that variations at these two SNPs were associated neither with persistence of HBV infection nor with HBV-induced liver abnormalities including cirrhosis or HCC in affected individuals.

We found that four haplotypes at the *MD-2* gene showed significantly different frequencies between HBV-infected patients and healthy controls. We found increased frequencies of the rs6472812-rs11466004 G-C and A-T haplotypes in cases compared to the haplotype frequencies found in the control group. We also found an increased frequency of the G-T haplotype in patients with cirrhosis + HCC compared to active carriers (0.240 versus 0.168, respectively; $P = 0.0436$), although we do consider the finding to be borderline significant. This haplotype includes the minor T allele of rs11466004 that was linked to an increased risk of progression to severe liver diseases although the association was not statistically significant.

We compared the allele frequency of SNPs studied in this report with other ethnic populations as per the 1000 genome project. We found that the allele frequency of *NOD2* SNP rs2066845 (G = 0.96, C = 0.04) was comparable to the allele frequency recorded among the population of Mexican ancestry (G = 0.98, C = 0.02), whereas the frequency of the alleles of SNP rs2066844 was close to those found in the Americans of black ancestry (C = 0.985, T = 0.015; G = 0.986, C = 0.014, respectively). On the other hand, the frequency of alleles of both rs6472812 (G = 61%, A = 39%) and rs11466004 (G = 58%, A = 42%) of *MD-2* was significantly higher than all populations recorded.

5. Conclusions

In conclusion, this study tested SNPs at the *NOD2* and *MD-2* genes and found evidence of association with susceptibility to HBV-infection in a Saudi Arabian population. Furthermore, the rs2066845 polymorphism at *NOD2* was associated with progression of HBV-related liver complications (cirrhosis and HCC) in Saudi patients with HBV. Further studies are needed to independently confirm the finding of this study and, more importantly, to identify the functional contributions of genetic variations in the *NOD2* and *MD-2* genes in HBV.

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