Association of single nucleotide polymorphisms in microRNAs with susceptibility to hepatitis B virus infection and HBV-related liver complications: A study in a Saudi Arabian population

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Summary

The aim of this study was to evaluate the association of 10 SNPs in different microRNAs (miRNAs) with susceptibility to hepatitis B virus (HBV) infection, HBV clearance, persistence of chronic HBV infection, and progression to liver cirrhosis and hepatocellular carcinoma (HCC). Patients were categorized into the following groups: inactive HBV carrier, active HBV carrier, HBV-cleared subject and cirrhosis+HCC. Samples were analysed for 10 SNPs in microRNAs using either PCR-based genotyping or the TaqMan assay. We found that rs1358379 was associated with susceptibility to HBV infection, HBV clearance, persistent chronic HBV infection and liver cirrhosis+HCC. In addition, we found that rs2292832 and rs11614913 were associated with risk of HBV infection, viral clearance and cirrhosis+HCC, whereas rs2910164 was associated with proneness to HBV infection, and ability to clear the virus. There was evidence of associations between rs6505162 and HBV clearance and the development of liver disease, whereas a single association was found between rs2289030 and HBV clearance. Similarly, rs7372209 and rs4919510 were specifically associated with the development of HBV-induced liver complications. SNPs in miRNAs affect the susceptibility, clearance and progression of HBV infection in Saudi Arabian patients. We found, using Gene Ontology or pathway analyses, that these genes may contribute to the
INTRODUCTION

Globally, one of the major causal agents of hepatitis is the Hepatitis B virus (HBV). It is estimated that approximately 240 million people worldwide have chronic hepatitis B infection.1 HBV infection can lead to liver cirrhosis, hepatic failure or hepatocellular carcinoma (HCC), which account for approximately 780,000 deaths each year globally.2 HCC is the fifth most common cancer and the third leading cause of cancer-related mortality in the world3 and it is estimated that approximately 53% of cases with HCC in the world are related to HBV.4

Molecular determinants for prognosis of HBV infection have yet to be identified. Several host and viral genetic factors are known to influence susceptibility to HBV infection, ability to clear the virus, maintenance of a chronic state and progression to advanced stages of HBV-related liver diseases. An important factor affecting different aspects of HBV infection that is gaining recognition are microRNAs (miRNAs). miRNAs are a group of endogenous, small noncoding RNAs, which are typically 20-22 nucleotides in length. They modulate an extensive array of biological processes, such as inflammatory responses, cell differentiation, proliferation and apoptosis.5 Although the mode of action of miRNAs is not fully understood, there is evidence that they act post-transcriptionally as negative regulators of gene expression through binding to target miRNAs regions, which results in mRNA cleavage or translational repression.7 There is increasing evidence that miRNAs regulate the expression of oncogenes and tumour suppressor genes, and consequently, their deregulation would have a crucial role in the instigation, progression and prognosis of various types of cancer.8–10

It has been proposed that single nucleotide polymorphisms (SNPs) found in miRNA genes affect miRNA transcription, processing and interactions with target miRNAs.11 Several studies have investigated SNPs in miRNA genes for evidence of association with HBV infection and the development of HCC. (Zhou et al.)12 performed a meta-analysis and found that miR-196a2 rs11614913 was associated with susceptibility to HBV infection,12 whereas no evidence of association was found between miR-146a rs2910164, miR-499 rs3746444, and miR-149 rs2292832 with hepatitis B risk. However, a different study found that miR-149 rs2292832 was associated with HBV-related HCC.13,14 In addition, Su et al. (2015)15 recently identified an association between miR-146a rs2910164 and the development of HCC in an Asian population. The lack of consistent findings supporting a role of miRNA SNPs in HBV infection and related complications may be because of several factors including genetic variations in the populations or ethnic groups studied and the number of samples (eg, cohort size) tested in these different studies.

In this study, we investigated ten SNPs in different miRNA genes (miR-499 rs3746444, miR-423 rs6505162, miR-26a1 rs7372209, miR-608 rs4919510, miR-604 rs2368392, miR-492 rs2289030, miR-149 rs2292832, miR-146a rs2910164, miR-196a2 rs11614913 and miR-30a rs1358379) for evidence of a role in the susceptibility, clearance and persistence of HBV infection within a Saudi Arabian population. This study identified evidence of association between several miRNA SNPs and HBV infection, prognosis and related liver complications.

MATERIALS AND METHODS

2.1 Subjects

A total of 1352 HBV-infected Saudi Arabian patients were recruited for this study. These patients were identified and subgrouped as follows: 585 were inactive asymptomatic HBV carriers, 222 were active symptomatic HBV carriers, 145 were HBV-infected patients diagnosed with liver cirrhosis or cirrhosis+HCC and 400 were HBV-cleared subjects. Furthermore, a group of 600 randomly selected, uninfected healthy individuals were recruited as controls. The study protocol conformed to the 1975 Declaration of Helsinki and was approved by the institutional review boards of three centres in Saudi Arabia, namely the King Faisal Specialist Hospital and Research Center in Riyadh, the Riyadh Military Hospital and the King Khalid University Hospital in Riyadh. Patients were recruited over a period of 3 years from August 2007 to August 2010. All patients signed informed consent prior to enrolling in the study, and their basic demographic data were recorded. Individuals who were positive for hepatitis B surface antigen (HBsAg) and negative for hepatitis B envelop antigen (HBeAg) and had normal to near-normal liver enzymes were considered to have a nonreplicative HBV infection and were grouped as inactive asymptomatic HBV carriers.16 Subjects, who were found to have repeated detection of HBsAg, were negative for HBeAg, and positive for antibodies to HBeAg (anti-HBe) with elevated serum ALT levels were grouped as active HBV carriers. Individuals from either of these groups showed no evidence of cirrhosis. Liver cirrhosis among patients with HBV infection was confirmed by liver biopsy. Diagnosis of HCC was performed using computed tomography and/or magnetic resonance imaging of liver according to guidelines published for the diagnosis and management of HCC.17 Control subjects were characterized by...
the absence of any known serological markers for HBV and by the lack of any evidence of liver disease.

2.2 | Genotyping miRNA single nucleotide polymorphisms

Genomic DNA from peripheral blood mononuclear cells was extracted using Gentra Puregene kit according to the manufacturer’s protocol (Qiagen, Hilden, Germany). Blood samples from patients and controls were genotyped for the miRNA SNPs using either a PCR-based genotyping assay or the TaqMan assay.

2.2.1 | PCR-based genotyping assay

Specific primers (forward 5'-AGAGTGAGGGAAAGGCACAA-3'; reverse 5'-GGGGTGAAGAGAAGCTGTA-3') for mir-499 rs3746444 were designed using Primer3 v.0.4.0 (http://frodo.wi.mit.edu/primer3). All PCR reactions were performed using the Veriti 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA). Amplified PCR products were separated on 2% agarose gels, using electrophoresis and visualized using ethidium bromide staining (0.5 μg/mL). DNA fragments were analysed by direct sequencing using a BigDye Terminator v3.1 Cycle Sequencing kit according to the manufacturer’s instructions (Applied Biosystems). Sequencing products were purified using DyeEx spin columns and eluted in 25 μL ddH₂O. Samples were vacuum-dried and resuspended in 15 μL of Hi-Di formamide. Samples were analysed using an ABI 3700 DNA Analyzer (Applied Biosystems).

2.2.2 | TaqMan genotyping assay

Nine miRNA SNPs—rs6505162, rs7372209, rs4919510, rs2368392, rs2910164, rs11614913 and rs1358379 were genotyped using the TaqMan allelic discrimination assay with a 7900 HT Fast Real Time PCR System (Applied Biosystems). TaqMan amplifying primers and probes were ordered from Applied Biosystems. For each SNP, one of the allelic probes was labelled with FAM and the other was labelled with VIC. PCR was performed using a TaqMan universal master mix (Applied Biosystems) at a probe concentration of 20×. Reactions were performed in a 96-well plate using 20 ng of genomic DNA in a total reaction volume of 25 μL. PCR cycling conditions were as follows: plates were first heated at 50°C for 2 minutes and then at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1.5 minutes. The fluorescence intensity of each well in the TaqMan assay plate was read. Fluorescence data files from each plate were analysed using automated software (SDS 2.4).

2.3 | Statistical analyses

Statistical analyses were performed using SPSS version 20 (SPSS Inc., Chicago, IL, USA). The genotype and allele distributions for each miRNA SNP in the patient groups, controls and clearance group were assessed using Pearson’s χ² test. Evidence of an association between SNPs and disease status was tested under additive, dominant and recessive genetic models, and results were expressed in terms of the odds ratio (OR) and 95% confidence interval (CI). A P value ≤.05 was considered statistically significant. To detect associations among categorical variables, we used chi-square statistics and one-way ANOVA to compare the means of continuous data. All miRNA genes investigated in this study were used as input core data for Genomatix Pathway Analysis (Genomatix, Munich, Germany).

3 | RESULTS

3.1 | General characteristics of the subjects

A total of 1352 HBV-infected patients and 600 healthy uninfected controls were analysed in this study. Baseline characteristics of the subjects are summarized in Table S1. The cases in this study included 585 inactive HBV carriers, 222 active HBV carriers, 145 HBV-infected patients with cirrhosis+HCC and 400 HBV-cleared individuals. All subjects were Saudi Arabian nationals.

3.2 | Genotyping

In the present analysis, 10 SNPs in different microRNA genes were investigated for evidence of an association with HBV infection and its related complications. These SNPs were miR-499 rs3746444, miR-423 rs6505162, miR-26a1 rs7372209, miR-608 rs4919510, miR-604 rs2368392, miR-492 rs2289030, miR-149 rs2292832, miR-146a rs2910164, miR-196a2 rs11614913 and miR-30a rs1358379. Genotype frequency distributions of all SNPs analysed in this study were in Hardy-Weinberg Equilibrium (data not shown).

Single nucleotide polymorphisms genotype distributions in controls and HBV-infected patients (excluding the HBV-cleared group) are shown in Table S2. We found that the SNPs rs2292832 (OR=3.86; 95% CI=3.15-4.73; P<.0001), rs2910164 (OR=0.724; 95% CI=0.618-0.847; P=.001), rs11614913 (OR=0.822; 95% CI=0.689-0.980; P=.029) and rs1358379 (OR=1.47; 95% CI=1.09-1.97; P=.011) were associated with hepatitis B infection in patients compared to that found in the control group. Furthermore, we found that individuals carrying either the homozygous rs2292832 TT genotype or the heterozygous CT genotype compared to individuals with the CC genotype were associated with an increased risk of HBV infection with an OR of 23.9 and 2.07, respectively. Comparably, the rs2292832 T allele was associated with HBV infection risk in both dominant (OR=3.26) and recessive models (OR=0.051). In addition, we found that rs2910164 and rs11614913 were associated with susceptibility to HBV infection under a dominant mode with an OR of 0.597 and 0.780, respectively (Table S2).

Next, these 10 polymorphisms were examined to determine whether they were associated with HBV clearance. Associations were identified between viral clearance and rs6505162 (OR=1.39, 95% CI=1.17-1.64; P=.0001), rs2289030 (OR=0.484; 95% CI=0.332-0.707; P<.0001), rs2910164 (OR=0.650; 95% CI=0.545-0.776; P<.0001), rs11614913 (OR=0.72; 95% CI=0.593-0.877; P=.001) and rs1358379 (OR=2.08; 95% CI=1.41-3.08; P<.001). Under a recessive mode of
inheritance. We found an increased frequency (0.61) of the rs6505162 C allele in HBV-cleared individuals compared to the frequency (0.53) found in patients with HBV infection (OR=0.353; 95% CI=0.246-0.505; *P*=.001). Similarly, increased frequencies of the rs2289030 C, rs2292832 C, rs2910164 C, rs11614913 T and rs1358379 T alleles were observed in individuals from the HBV-cleared group compared to that found in the HBV-infected group. We also found that the rs2289030 C allele, rs2292832 C allele and rs2910164 C allele were predictive of HBV clearance. In addition, the rs11614913 T allele was associated with HBV clearance in both dominant (OR=0.743; 95% CI=0.586-0.943; *P*=.014) and recessive models (OR=2.488; 95% CI=1.466-4.221; *P*=.0005). A similar association was found between the rs1358379 T allele under a recessive mode of inheritance (OR=0.031; 95% CI=0.002-0.512; *P*=.0001) (Table 1).

Allele and genotype frequency comparisons were performed between patients from the active carrier and cirrhosis/HCC groups with the inactive HBV carrier group. We identified rs2292832 and rs1358379 that were associated with HBV persistence (OR=1.217; 95% CI=1.001-1.479; *P*=.049 and OR=3.615; 95% CI=2.542-5.141; *P*<.0001). Specifically, we found that the rs1358379 homozygous CC genotype was associated with HBV persistence (OR=14.52; 95% CI=5.09-41.45; *P*<.0001), with similar findings observed with the rs1358379 C allele using a recessive model (OR=0.072, 95% CI=0.025-0.206; *P*=.0001) and dominant model (OR=2.762, 95% CI=1.855-4.112; *P*<.0001). These results indicate a putative role of the homozygous CC genotype of rs1358379 towards maintaining a continuous HBV infection state in patients (Table S3).

We also examined the potential role of these miRNA SNPs in the progression of HBV infection to more severe stages of liver disease. Six SNPs showed a significant association with development of cirrhosis or cirrhosis+HCC among HBV-infected patients compared to that found in active HBV carriers (Table 2). The polymorphisms rs7372209 (OR=1.71; 95% CI=1.023-2.861; *P*=.039), rs4919510 (OR=1.93; 95% CI=1.364-2.72; *P*<.0002), rs2292832 (OR=1.94; 95% CI=1.43-2.64; *P*<.0001) and rs1358379 (OR=13.38; 95% CI=7.43-24.095; *P*<.0001) were associated with an increased risk of disease progression in patients with HBV. We found that the rs7372209 T allele was significantly associated with more severe outcomes of HBV infection under a dominant model (OR=1.884; 95% CI=1.075-3.301; *P*=.02), with similar findings observed in individuals with the homozygous CT genotype (OR=1.974; 95% CI=1.102-3.535; *P*<.025). Similarly, evidence of associations was found between disease severity and individuals with the homozygous rs4919510 GG genotype (OR=6.072; 95% CI=2.496-14.776; *P*<.0001) and the rs4919510 G-allele in both recessive (OR=0.173; 95% CI=0.072-0.414; *P*<.0001) as well as dominant genetic models (OR=1.605; 95% CI=1.045-2.464; *P*=.030). We also found that individuals with the homozygous rs2292832 TT genotype were associated with HBV infection complications (OR=2.580; 95% CI=1.511-4.405; *P*<.001), with comparable support found with the rs2292832 T allele using a dominant (OR=1.985; 95% CI=1.298-3.036; *P*=.0015) or recessive mode of inheritance (OR=0.459; 95% CI=0.278-0.757; *P*=.002). The C allele of rs11614913 was associated with cirrhosis+HCC solely under a dominant model (OR=0.613; 95% CI=0.394-0.953; *P*=.03), whereas the rs1358379 C allele under a dominant model (OR=9.348; 95% CI=4.949-17.660; *P*<.0001) and individuals with the heterozygous rs1358379 CT genotype (OR=4.006; 95% CI=1.981-8.103; *P*<.0001) were associated with an increased risk of complications. In contrast, individuals with the homozygous rs1358379 CC genotype (OR=151.425; 95% CI=9.171-2500.125; *P*<.0001) and the rs1358379 C allele using a recessive model (OR=0.008; 95% CI=0.000-0.129; *P*<.0001) were associated with increased disease severity. However, this finding should be interpreted with caution, as there were no active HBV carriers with the homozygous rs1358379 CC genotype. We found that the rs6505162 polymorphism was associated with the development of severe diseases in HBV-infected patients (OR=0.617; 95% CI=0.456-0.834; *P*=.002). More specifically, using a dominant model, the rs6505162 A allele was associated with a reduced risk for development of cirrhosis+HCC (OR=0.432; 95% CI=0.275-0.678; *P*<.0001). Similarly, individuals with the homozygous rs6505162 AA genotype (OR=0.434; 95% CI=0.239-0.790; *P*<.0006) or heterozygous AC genotype (OR=0.434; 95% CI=0.265-0.699; *P*<.0001) were associated with a decreased risk for development of severe liver diseases. These findings indicate that the rs6505162 A allele may have a protective role against the development of complications in HBV-infected patients (Table 2).

### 4 DISCUSSION

In this study, 10 SNPs in different miRNAs were analysed to determine whether there was evidence of an association with HBV susceptibility, the progression of HBV-related liver diseases or viral clearance in recovered individuals. Several previous studies investigated miR-30a rs1358379 for association with different carcinomas. However, there is a lack of data to correlate rs1358379 alleles or genotypes with susceptibility to HBV infection and the risk of related liver diseases. In the present study, we found support of an association between the rs1358379 C allele with susceptibility to HBV infection. It was also determined that the T allele of this SNP is associated with an increased ability of HBV-infected patients to clear the virus. In addition, miR-30a rs1358379 was the only polymorphism tested in this study that was associated with HBV persistence. Upon further analysis to determine the effect of miR-30a rs1358379 on disease severity, we found that the rs1358379 C allele was associated with an increased risk of developing cirrhosis and HCC compared to that found in active HBV carriers. Budhu et al. investigated the role of miRNAs in metastatic HCC and found that miR-30a was downregulated, and consequently, its targets were upregulated. In this context, we propose that rs1358379 modulates the expression of mature miR-30a that results in changes in target genes, which are involved in HCC development.

The SNPs miR-149 rs2292832 and miR-196a2 rs11614913 have been widely tested to determine their possible roles in various types of cancer. We found that rs2292832 was associated with an increased risk of HBV infection and progression to cirrhosis and HCC. Our findings are consistent with those of previous studies that found an association of miR-149 C>T with HBV-related HCC.
| Gene  | SNPs    | Genotype/Allele distribution | Clearance n=400 (%) | Patients n=952 (%) | OR (95% C.I.) | $\chi^2$ | P-value |
|-------|---------|-----------------------------|---------------------|-------------------|--------------|---------|---------|
| mir-499 | rs3746444 | TT                          | 148 (37)            | 354 (37.2)        | Ref          |         |         |
|       |         | CT                          | 187 (46.75)         | 443 (46.5)        | 0.990 (0.766-1.281) | 0.01    | .9410   |
|       |         | CC                          | 65 (16.25)          | 155 (16.3)        | 0.997 (0.704-1.411) | 0.00    | .9860   |
|       |         | T                           | 483 (60.4)          | 1151 (60.45)      | 0.997 (0.842-1.180) | 0.00    | .9703   |
|       |         | C                           | 317 (39.6)          | 753 (39.55)       |              |         |         |
|       |         | CC vs TT+CT                 | 0.998 (0.727-1.369) | 0.01              | .9890        |         |         |
|       |         | CC+CT vs TT                 | 0.992 (0.779-1.264) | 0.00              | .9490        |         |         |
| mir-423 | rs6505162 | CC                          | 128 (32)            | 285 (30)          | Ref          |         |         |
|       |         | AC                          | 232 (58)            | 439 (46)          | 0.850 (0.654-1.105) | 1.48    | .2240   |
|       |         | AA                          | 40 (10)             | 228 (24)          | 2.560 (1.724-3.802) | 22.58   | <.0001  |
|       |         | C                           | 488 (61)            | 1009 (53)         | 1.387 (1.173-1.641) | 14.61   | <.0001  |
|       |         | A                           | 312 (39)            | 895 (47)          |              |         |         |
|       |         | AA vs CC+AC                 | 0.353 (0.246-0.505) | 34.49             | <.0001       |         |         |
|       |         | AA+AC vs CC                 | 1.101 (0.856-1.417) | 0.57              | .4520        |         |         |
| mir-26a1 | rs7372209 | CC                          | 309 (77.25)         | 778 (81.7)        | Ref          |         |         |
|       |         | CT                          | 84 (21)             | 158 (16.6)        | 0.747 (0.556-1.004) | 3.75    | .0530   |
|       |         | TT                          | 7 (1.75)            | 16 (1.7)          | 0.908 (0.370-2.228) | 0.04    | .8330   |
|       |         | C                           | 702 (87.75)         | 1714 (90)         | 0.794 (0.613-1.029) | 3.05    | .0800   |
|       |         | T                           | 98 (12.25)          | 190 (10)          |              |         |         |
|       |         | TT vs CC+CT                 | 1.042 (0.425-2.553) | 0.01              | .9280        |         |         |
|       |         | TT+CT vs CC                 | 0.759 (0.571-1.011) | 3.58              | .0590        |         |         |
| mir-608 | rs4919510 | CC                          | 248 (62)            | 594 (62.4)        | Ref          |         |         |
|       |         | CG                          | 128 (32)            | 293 (30.8)        | 0.956 (0.741-1.233) | 1.12    | .7280   |
|       |         | GG                          | 24 (6)              | 65 (6.8)          | 1.131 (0.692-1.848) | 0.24    | .6240   |
|       |         | C                           | 624 (78)            | 1481 (77.8)       | 1.013 (0.830-1.236) | 0.02    | .9010   |
|       |         | G                           | 176 (22)            | 423 (22.2)        |              |         |         |
|       |         | GG vs CC+CG                 | 0.871 (0.537-1.412) | 0.31              | .5750        |         |         |
|       |         | GG+CG vs CC                 | 0.983 (0.773-1.251) | 0.02              | .8910        |         |         |
| mir-604 | rs2368392 | GG                          | 245 (61.25)         | 627 (66)          | Ref          |         |         |
|       |         | AG                          | 155 (38.75)         | 325 (34)          | 0.819 (0.643-1.043) | 2.62    | .1060   |
|       |         | AA                          | 0 (0)               | 0 (0)             | 0.391 (0.008-19.772) | nan     | 1.000   |
|       |         | G                           | 645 (80.6)          | 1579 (83)         | 0.857 (0.693-1.059) | 2.05    | .1520   |
|       |         | A                           | 155 (19.4)          | 325 (17)          |              |         |         |
|       |         | AA vs GG+AG                 | 2.378 (0.047-120.066) | nan              | 1.000        |         |         |
|       |         | AA+AG vs GG                 | 0.819 (0.643-1.043) | 2.62              | .1060        |         |         |
| mir-492 | rs2289030 | GG                          | 349 (87.25)         | 892 (93.7)        | Ref          |         |         |
|       |         | CG                          | 50 (12.5)           | 58 (6.1)          | 0.454 (0.305-0.676) | 15.75   | <.0001  |
|       |         | CC                          | 1 (0.25)            | 2 (0.2)           | 0.783 (0.071-8.657) | 0.04    | .8410   |
|       |         | G                           | 748 (93.5)          | 1842 (96.7)       | 0.484 (0.332-0.707) | 14.68   | <.0001  |
|       |         | C                           | 52 (6.5)            | 62 (3.3)          |              |         |         |
|       |         | CC vs GG+CG                 | 1.190 (0.108-13.166) | 0.02              | .8870        |         |         |
|       |         | CC+CG vs GG                 | 0.460 (0.311-0.682) | 15.54             | <.0001       |         |         |

(Continues)
found that the miR-149 rs2292832 C allele under a recessive model was associated with improved viral clearance and was found at an increased frequency in HBV-cleared patients. These findings suggest that the miR-149 rs2292832 homozygous CC genotype may confer a protective effect against HBV-related liver disease. In addition, Kim et al.\textsuperscript{14} reported an association between the C allele of miR-149 rs2292832 and reduced risk of HCC. We found under a dominant genetic model that the miR-196a2 rs11614913 C allele was associated with increased susceptibility to HBV infection, with a similar association found for individuals with the homozygous CC genotype. Our results concur with a recent analysis.\textsuperscript{12} However, the risk T allele of this SNP was associated with HBV clearance under a dominant and recessive mode of inheritance, a finding that suggests that individuals with a single copy of the T allele are at a reduced risk of progressing to a chronic HBV infection. Previous studies have reported conflicting results regarding the role of miR-196a-2 rs11614913 with susceptibility to cirrhosis and HCC in HBV-infected patients. Han et al.\textsuperscript{24} did not find any evidence of an association with risk of HBV-related HCC in a Chinese population. However, a study that investigated the role of rs11614913 in HCC progression in a Turkish population found an association between the homozygous CC genotype and HBV-related HCC risk.\textsuperscript{23} This finding is in accordance with our study in which we found that the rs11614913 homozygous CC genotype in a dominant mode of inheritance was associated with increased susceptibility to HCC in our Saudi Arabian population. Several studies reported that the miR-196a-2 rs11614913 CC genotype was associated with increased expression of mature miR-196a-2.\textsuperscript{25,26} Schimanski et al.\textsuperscript{27} demonstrated that high levels of miR-196a-2 exerted an oncogenic potential promoting cancer cell migration and invasion.

The rs2910164 polymorphism, which is located in the miR-146a gene, is associated with several types of cancer.\textsuperscript{7} We found that the rs2910164 G-allele was associated with an increased risk of HBV

### TABLE 1 (Continued)

| Gene     | SNPs   | Genotype/Allele distribution | Clearance n=400 (%) | Patients\textsuperscript{3} n=952 (%) | OR (95% C.I.) | $\chi^2$ | P-value |
|----------|--------|-------------------------------|---------------------|--------------------------------------|---------------|---------|---------|
| mir-149  | rs2292832 | CC 208 (52) | 505 (53) | Ref                                 |
|          |        | CT 136 (34) | 268 (28.2) | 0.812 (0.625-1.055) | 2.44 | .1182 |
|          |        | TT 56 (14)  | 179 (18.8) | 1.317 (0.936-1.851) | 2.51 | .1130 |
|          |        | C 552 (69)  | 1278 (67.1) | 1.090 (0.913-1.302) | 0.91 | .3400 |
|          |        | T 248 (31)  | 626 (32.9)  |                          |       |        |
|          |        | TT vs CC+CT | 0.703 (0.507-0.974) | 4.52 | .0330 |
|          |        | TT+CT vs CC | 0.959 (0.759-1.212) | 0.12 | .7250 |
|          | rs2910164 | GG 155 (38.75) | 522 (54.8) | Ref                                 |
|          |        | CG 202 (50.5) | 350 (36.8) | 0.514 (0.401-0.660) | 27.69 | <.0001 |
|          |        | CC 43 (10.75) | 80 (8.4)  | 0.552 (0.366-0.834) | 8.13 | .0040 |
|          |        | G 512 (64)  | 1394 (73.2) | 0.650 (0.545-0.776) | 22.99 | <.0001 |
|          |        | C 288 (36)  | 510 (26.8)  |                          |       |        |
|          |        | CC vs GG+CG | 1.313 (0.888-1.940) | 1.88 | .1710 |
|          |        | CC+CG vs GG | 0.521 (0.411-0.661) | 29.14 | <.0001 |
|          | rs11614913 | CC 228 (57) | 610 (64)  | Ref                                 |
|          |        | CT 143 (35.75) | 313 (33) | 0.818 (0.637-1.050) | 2.49 | .1150 |
|          |        | TT 29 (7.25) | 29 (3)  | 0.374 (0.219-0.639) | 13.78 | .0002 |
|          |        | C 599 (74.9) | 1533 (80.5) | 0.721 (0.593-0.877) | 10.74 | .0010 |
|          |        | T 201 (25.1) | 371 (19.5)  |                          |       |        |
|          |        | TT vs CC+CT | 2.488 (1.466-4.221) | 12.12 | .0005 |
|          |        | TT+CT vs CC | 0.743 (0.586-0.943) | 5.98 | .0140 |
|          | rs1358379 | TT 368 (92) | 836 (87.8) | Ref                                 |
|          |        | CT 32 (8) | 80 (8.4)  | 1.100 (0.717-1.688) | 0.19 | .6610 |
|          |        | CC 0 (0)  | 36 (3.8)  | 32.158 (1.968-525.364) | 15.65 | <.0001 |
|          |        | T 768 (96) | 1752 (92) | 2.082 (1.409-3.077) | 14.09 | .0002 |
|          |        | C 32 (4)  | 152 (8)  |                          |       |        |
|          |        | CC vs TT+CT | 0.031 (0.002-0.512) | 15.54 | <.0001 |
|          |        | CC+CT vs TT | 1.596 (1.059-2.405) | 5.06 | .0240 |

\textsuperscript{a}Patients are categorized to Inactive, Active, cirrhosis and hepatocellular carcinoma.
| Gene     | SNPs   | Genotype/Allele distribution | Active n=222 (%) | Cirr+HCC n=145 (%) | OR (95% C.I.) | \( \chi^2 \) | P-value |
|----------|--------|-----------------------------|------------------|---------------------|---------------|-------------|---------|
| mir-499  | rs3746444 | TT                          | 87 (39.19)       | 48 (33.1)           | Ref           |             |         |
|          |        | CT                          | 100 (45.05)      | 70 (48.28)          | 1.269 (0.796-2.023) | 1.00       | .3170   |
|          |        | CC                          | 35 (15.77)       | 27 (18.62)          | 1.398 (0.757-2.582) | 1.15       | .2830   |
|          |        | T                           | 274 (61.71)      | 166 (57.24)         | 1.204 (0.891-1.627) | 1.16       | .2270   |
|          |        | C                           | 170 (38.29)      | 124 (42.76)         |               |             |         |
|          |        | CC vs TT+CT                 |                  |                     | 0.818 (0.471-1.421) | 0.51       | .4750   |
|          |        | CC+CT vs TT                 |                  |                     | 1.302 (0.840-2.019) | 1.40       | .2370   |
| mir-423  | rs6505162 | CC                          | 53 (23.87)       | 61 (42.07)          | Ref           |             |         |
|          |        | AC                          | 117 (52.7)       | 58 (40)             | 0.434 (0.265-0.699) | 11.82      | .0006   |
|          |        | AA                          | 52 (23.42)       | 26 (17.93)          | 0.434 (0.239-0.790) | 7.61       | .0060   |
|          |        | C                           | 223 (50.23)      | 180 (62.07)         | 0.617 (0.456-0.834) | 9.94       | .0020   |
|          |        | A                           | 221 (49.77)      | 110 (37.93)         |               |             |         |
|          |        | AA vs CC+AC                 |                  |                     | 1.400 (0.827-2.369) | 1.58       | .2090   |
|          |        | AA+AC vs CC                 |                  |                     | 0.432 (0.275-0.678) | 13.56      | .0002   |
| mir-26a1 | rs7372209 | CC                          | 194 (87.39)      | 114 (78.62)         | Ref           |             |         |
|          |        | CT                          | 25 (11.26)       | 29 (20)             | 1.974 (1.102-3.535) | 5.36       | .0210   |
|          |        | TT                          | 3 (1.35)         | 2 (1.38)            | 1.135 (0.187-6.891) | 0.02       | .8910   |
|          |        | C                           | 413 (93.02)      | 257 (88.62)         | 1.711 (1.023-2.861) | 4.26       | .0390   |
|          |        | T                           | 31 (6.98)        | 33 (11.38)          |               |             |         |
|          |        | TT vs CC+CT                 |                  |                     | 0.979 (0.162-5.935) | 0.00       | .9820   |
|          |        | TT+CT vs CC                 |                  |                     | 1.884 (1.075-3.301) | 5.00       | .0250   |
| mir-608  | rs4919510 | CC                          | 146 (65.77)      | 79 (54.48)          | Ref           |             |         |
|          |        | CG                          | 69 (31.08)       | 43 (29.66)          | 1.152 (0.721-1.841) | 0.35       | .5550   |
|          |        | GG                          | 7 (3.15)         | 23 (15.86)          | 6.072 (2.496-14.776) | 19.05      | <.0001  |
|          |        | C                           | 361 (81.31)      | 201 (69.31)         | 1.926 (1.364-2.720) | 14.07      | .0002   |
|          |        | G                           | 83 (18.69)       | 89 (30.69)          |               |             |         |
|          |        | GG vs CC+CG                 |                  |                     | 0.173 (0.072-0.414) | 18.87      | <.0001  |
|          |        | GG+CG vs CC                 |                  |                     | 1.605 (1.045-2.464) | 4.71       | .0300   |
| mir-604  | rs2368392 | GG                          | 152 (68.47)      | 102 (70.34)         | Ref           |             |         |
|          |        | AG                          | 70 (31.53)       | 43 (29.66)          | 0.915 (0.581-1.443) | 0.14       | .7030   |
|          |        | AA                          | 0 (0)            | 0 (0)               | 1.488 (0.029-75.582) | nan 1.00   |
|          |        | G                           | 374 (84.23)      | 247 (85.17)         | 0.930 (0.616-1.405) | 0.12       | .7310   |
|          |        | A                           | 70 (15.77)       | 43 (14.83)          |               |             |         |
|          |        | AA vs GG+AG                 |                  |                     | 0.654 (0.013-33.140) | nan 1.00   |
|          |        | AA+AG vs GG                 |                  |                     | 0.915 (0.581-1.443) | 0.14       | .7030   |
| mir-492  | rs2289030 | GG                          | 206 (92.79)      | 138 (95.17)         | Ref           |             |         |
|          |        | CG                          | 16 (7.21)        | 7 (4.83)            | 0.653 (0.262-1.629) | 0.85       | .3580   |
|          |        | CC                          | 0 (0)            | 0 (0)               | 1.491 (0.029-75.585) | nan 1.00   |
|          |        | G                           | 428 (96.4)       | 283 (97.59)         | 0.662 (0.269-1.629) | 0.82       | .3660   |
|          |        | C                           | 16 (3.6)         | 7 (2.41)            |               |             |         |
|          |        | CC vs GG+CG                 |                  |                     | 0.654 (0.013-33.140) | nan 1.00   |
|          |        | CC+CG vs GG                 |                  |                     | 0.653 (0.262-1.629) | 0.85       | .3580   |
(Continues)
infection. In contrast, a recent meta-analysis did not find any significant correlation between the miR-146a rs2910164 G > C polymorphism and hepatitis B susceptibility. This disparity may be because of the comparatively small sample sizes tested in the studies examined in this meta-analysis. In the present study, we found that the risk C allele under a dominant model was associated with HBV clearance. Therefore, this finding indicates that inheriting a single rs2910164 C allele would reduce an individual’s risk of developing a chronic HBV infection. Xu et al. demonstrated that the C allele was associated with decreased production of mature miR-146a compared to that found with the G-allele. Li et al. reported a positive correlation between miR-146a expression and HBV replication level, while another recent study found that the miR-146 family was responsible for enhanced HBV replication.

TABLE 2 (Continued)

| Gene | SNPs | Genotype/Allele distribution | Active n=222 (%) | Cirr+HCC n=145 (%) | OR (95% C.I.) | χ² | P-value |
|------|------|-------------------------------|-----------------|-------------------|--------------|----|--------|
| mir-149 | rs2292832 | CC | 128 (57.66) | 59 (40.69) | Ref | | |
| | | CT | 57 (25.68) | 42 (28.97) | 1.599 (0.966-2.646) | 3.35 | .0670 |
| | | TT | 37 (16.67) | 44 (30.34) | 2.580 (1.511-4.405) | 12.38 | .0004 |
| | | T | 131 (29.5) | 160 (55.17) | 1.941 (1.426-2.643) | 17.97 | <.0001 |
| | | TT vs CC+CT | 0.459 (0.278-0.757) | 9.54 | .0020 |
| | | TT+CT vs CC | 1.985 (1.298-3.036) | 10.10 | .0015 |
| mir-146a | rs2910164 | GG | 120 (54.05) | 92 (63.45) | Ref | | |
| | | CG | 82 (36.94) | 40 (27.59) | 0.636 (0.399-1.013) | 2.65 | .0560 |
| | | CC | 20 (9.01) | 13 (8.97) | 0.848 (0.401-1.793) | 0.19 | .6650 |
| | | G | 322 (72.52) | 224 (77.24) | 0.778 (0.551-1.098) | 2.05 | .1520 |
| | | C | 122 (27.48) | 66 (22.76) | 1.005 (0.484-2.090) | 0.00 | .9890 |
| | | CC vs GG+CG | 0.678 (0.441-1.041) | 3.17 | .0750 |
| mir-196a2 | rs11614913 | CC | 128 (57.66) | 100 (68.77) | Ref | | |
| | | CT | 84 (37.84) | 39 (26.9) | 0.594 (0.375-0.942) | 4.93 | .0260 |
| | | TT | 10 (4.5) | 6 (4.14) | 0.768 (0.270-2.185) | 0.25 | .6190 |
| | | T | 104 (23.42) | 112 (38.62) | 0.698 (0.480-1.014) | 3.59 | .0580 |
| | | TT vs CC+CT | 1.093 (0.388-3.075) | 0.03 | .8660 |
| | | TT+CT vs CC | 0.613 (0.394-0.953) | 4.77 | .0290 |
| mir-30a | rs1358379 | TT | 208 (93.69) | 89 (61.38) | Ref | | |
| | | CT | 14 (6.31) | 24 (16.55) | 4.006 (1.981-8.103) | 16.60 | <.0001 |
| | | CC | 0 (0) | 32 (22.07) | 151.425 (9.171-2500.125) | 60.94 | <.0001 |
| | | T | 430 (96.85) | 202 (69.66) | 13.380 (7.430-24.095) | 108.40 | <.0001 |
| | | CC vs TT+CT | 0.008 (0.000-0.129) | 53.67 | <.0001 |
| | | CC+CT vs TT | 9.348 (4.949-17.660) | 59.34 | <.0001 |

The SNP rs6505162 in the miR-423 gene has been previously associated with different types of cancer. We did not find evidence of an association between rs6505162 and susceptibility of individuals to HBV infection. However, we found using a dominant model that the rs6505162 C allele was associated with an increased ability of individuals to clear the virus. Li et al. evaluated miRNAs as serum biomarkers for HBV-related HCC and did not find an association between miR-423 and development of HCC. In contrast, we found that the miR-423 rs6505162 A allele was associated with a decreased risk of cirrhosis+HCC in HBV-infected patients. Similarly, individuals with the homozygous AA or heterozygous AC genotype were significantly associated with reduced progression of HCC compared to that found in individuals with the homozygous CC genotype, a finding which indicates that the A allele may have a protective effect against disease progression. Our findings are comparable to previous studies that reported a protective role of rs6505162 in breast cancer.
MiR-26a1 rs7372209 is an understudied polymorphism in HBV infection and its complications. We found that the rs7372209 T allele under a dominant model was specifically associated with progression to cirrhosis+HCC in HBV-infected patients. Previous studies have reported that mir-26a1 inhibits cancer cell proliferation via proposed mechanisms including induction of cell cycle arrest in HCC cells. However, the functional significance and role of this SNP on miR-26a1 expression is not known. Stenholm et al. evaluated the effects of miRNA polymorphisms on prognosis of advanced gastric cancer and proposed that the T allele of rs7372209 was associated with impaired mir-26a1 expression or function. This proposal is consistent with our results in that we posit low expression of miR-26a1 has a subsequent effect on regulation of cell immunity.

A previous study found that rs4919510 in the miR-608 gene affects the secondary structure and ancestral miR-608 stem-loop sequence, thereby modulating the ability of the microRNA to bind to potential targets. In the present study, we found evidence of an association with the development of cirrhosis and HCC in HBV-infected patients. The rs4919510 G-allele under all three genetic models (additive, dominant and recessive) was associated with an increased risk of liver cirrhosis and HCC. Several studies have linked miR-608 rs4919510 with different cancers such as nasopharyngeal carcinoma, colorectal cancer and breast cancer. Although we speculate that a comparable mechanism underlies the role of the rs4919510 G-allele and increased risk of HCC, further experimental evidence is required.

In this study, enrichment analysis of these 10 microRNA genes generated two functional pathways, HBV and HCC. The presence of these genes in these functional pathways supports their association with HBV and HCC. Disease pathway analysis predicted that mir-146a, mir-26a1, mir-30a, mir196a2, mir499a and mir-149 are involved in HBV infection (Figure 1), a prediction confirmed by our findings. Similarly, mir-30a, mir-146a, mir499a, mir-26a1, mir196a2, mir423 and mir-604 are involved in the disease pathway for HCC (Fig. S1). These microRNAs may have direct or indirect effects on several regulatory molecules that directly bind to DNA sequences in the nucleus. However, further experiments are required to elucidate the exact role of these genes in the pathogenesis of HBV infection and its complications.
In conclusion, this investigation demonstrated that SNPs in different microRNA genes influence susceptibility, clearance and progression of HBV infection within a Saudi Arabian population. With the exception of miR-499 rs3746444 and miR-604 rs2368392, we found evidence of association between eight SNPs with one or more clinical stage of HBV infection (Figure 2). Following confirmation in a larger sample size, our findings may be used to identify individuals at an increased risk of HBV infection and HCC, and in particular, individuals from the Saudi Arabian population where the availability of such information is limited. Unfortunately, testing to determine the functional significance and effects of these miRNA polymorphisms was beyond the scope of this study. However, our findings may pave the way for future experiments to elucidate the roles of these miRNA SNPs in the mechanism that underlies regulation of those pathways involved in HBV infection and its related complications, and towards the identification of novel targets for therapeutic approaches.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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**SUPPORTING INFORMATION**

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