



Association of Polymorphism in MicroRNA 604 with Susceptibility to Persistent Hepatitis B Virus Infection and Development of Hepatocellular Carcinoma

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MicroRNA polymorphisms may be associated with carcinogenesis or immunopathogenesis of infection. We evaluated whether the microRNA-604 (miR-604) polymorphism can affect the persistence of hepatitis B virus (HBV) infection, and the development to hepatocellular carcinoma (HCC) in patients with chronic HBV infection. A total of 1,439 subjects, who have either past or present HBV infection, were enrolled and divided into four groups (spontaneous recovery, chronic HBV carrier without cirrhosis, liver cirrhosis and HCC). We genotyped the precursor miR-604 genome region polymorphism. The CC genotype of miR-604 rs2368392 was most frequently observed and T allele frequency was 0.326 in all study subjects. The HBV persistence after infection was higher in those subjects with miR-604 T allele ($P = 0.05$ in a co-dominant and dominant model), which implied that the patients with miR-604 T allele may have a higher risk for HBV chronicity. In contrast, there was a higher rate of the miR-604 T allele in the chronic carrier without HCC patients, compared to those of the HCC patients ($P = 0.03$ in a co-dominant model, $P = 0.02$ in a recessive model). The T allele at miR-604 rs2368392 may be a risk allele for the chronicity of HBV infection, but may be a protective allele for the progression to HCC in chronic HBV carriers.

Keywords: Hepatitis B Virus; Carcinoma, Hepatocellular; MicroRNAs; Polymorphism

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most commonly diagnosed cancers and the third leading cause of cancer-related deaths worldwide (1). Chronic infection of hepatitis B virus (HBV) and hepatitis C virus (HCV) are the most important causes of cirrhosis and HCC. In Korea, HBV is the leading cause of cirrhosis and HCC (2). Screening with ultrasound and alpha fetoprotein is hindered by suboptimal sensitivity and specificity. Thus, identification of markers that are associated with an increased risk of HCC would better define the populations with highest risk for HCC.

Increasing evidence indicates that genetic variation in the regulatory regions could be a major contributor to phenotypic diversity in the human population (3). MicroRNAs (miRNAs) are a class of single-stranded non-coding RNAs of 19-25 nucleotides, in length, in their mature form that act as posttranscriptional regulators of gene expression (4). MiRNA has emerged as novel players in the control of genes expression in cancer. It has been hypothesized that polymorphisms in pre-miRNA may in-

fluence the miRNA maturation, and thereby, modulate miRNA expression. Because each miRNA has numerous targets, inherited minor variations in miRNA expression could have important consequences on the expression of various protein-coding oncogenes and tumor suppressors. Recently, a pre-miRNA single nucleotide polymorphism (SNP) in miR-196a2 was found to be associated with survival in individuals with non-small cell lung cancer (5). Another case-control study in Chinese women, with breast cancer, identified the rs11614913 (T > C) polymorphism in miR-196a2 and an A > G SNP (rs3746444) in miR-499 were associated with a significantly increased risk of breast cancer susceptibility (6).

Pre-miRNA polymorphism, which is associated with cancers, has been reported, but studies on miR-604 are rare. The association between miR-604 SNP and cancer development or recurrence or survival was recently performed in patients with renal cell carcinoma (7) and oropharyngeal cancer (8), as well as colon cancer (9), but they showed negative results. To date, the role of miR-604 genetic variants in HCC susceptibility and disease progression in patients with HBV infection has not been

reported. We thought that it is worthy to elucidate whether miR-604 SNP is associated with the susceptibility to HCC development, because microRNA can affect to tumorigenesis in organ specific manner.

To identify genetic factors, that are associated with HBV-related HCC and progression of HBV-related chronic liver disease, we conducted an association study that analyzes miR-604 SNP in DNA isolated from peripheral blood.

MATERIALS AND METHODS

Study patients

To serve as primary patients for the case-control study, a total of 1,439 individuals, having either past or present evidence of HBV infection, were recruited from the outpatient clinic of the liver unit and the Center for Health Promotion Center at Seoul National University Hospital, as well as Ajou University Hospital. Spontaneously recovered subjects were defined by a repeated seropositivity for both anti-HBs (antibody to hepatitis B surface antigen) and anti-HBc (antibody to hepatitis B core antigen) of the IgG type without HBsAg (10). The chronic carrier group was evaluated further for disease progression to cirrhosis or occurrence of HCC. Patients with chronic HBV infection had followed up regularly with serum alpha-fetoprotein level, abdominal ultrasonography, and/or a 4-phase spiral liver CT scan of more than twice a year for HCC screening. Dynamic contrast enhanced abdominal MRI, bone scans, chest CT, brain MRI, brain CT, hepatic angiography, or PET scan was also carried out in selected cases based on the clinical requirement.

Diagnosis of HCC was based on imaging findings of nodules that were larger than 1 cm, showing intense arterial uptake, followed by washout of contrast in the venous-delayed phases, in a 4-phase multi-detector CT scan or dynamic contrast enhanced MRI and/or biopsy (11). Cirrhosis of the liver was diagnosed either by liver biopsy or based on the clinical evidence of portal hypertension (esophageal varices, palpable splenomegaly and definite findings of cirrhotic liver or ascites by ultrasonography). The age of onset of HCC was determined, according to the date of initial diagnosis.

Exclusion criteria for the study patients included the following: 1) tested positive for anti-HBs, but not for anti-HBc; 2) tested positive for anti-HCV or anti-HIV (GENEDIA[®]; Greencross Life Science Corp., Yongin-shi, Korea, HCV[®]3.2; Dong-A Pharmaceutical Co., Seoul, Korea); 3) average alcohol consumption of ≥ 10 g/day or an average number of ≥ 1 cigarette pack(s) smoked daily, which was assessed through individual interviews; and 4) incurrance of other types of liver diseases, such as autoimmune hepatitis, toxic hepatitis, hemochromatosis, Wilson's disease, non-alcoholic steatohepatitis, primary biliary cirrhosis, or Budd-Chiari syndrome. None of the patients had previous history of immunosuppressant or anti-viral treatment.

Genotyping of miR-604 genome region polymorphism, rs2368392

Using the Wizard genomic DNA purification kit (Promega, Madison, WI, USA), genomic DNA was extracted from patients' peripheral blood samples. SNP genotyping was performed using the TaqMan[®] (12) assay in the ABI prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA), as previously described (10). Genotyping quality control was performed in 10% of the samples, by duplicate checking (rate of concordance in duplicates = 100%). Assay IDs of rs2368392 was 'C_16218215_10' (Applied Biosystems).

Statistical analyses

To determine the association of rs2368392, with hepatitis B virus (HBV) clearance, and hepatocellular carcinoma (HCC) occurrence, the odds ratio (95% confidence interval) was calculated using logistic analysis adjusted for age (continuous value) and sex (male = 0, female = 1), as covariates to eliminate or reduce any confounds that might influence the findings. Data was managed and analyzed, using the Statistical Analysis System (SAS) version 9.1 (SAS Inc., Cary, NC, USA).

Ethics statement

The study protocol was reviewed and approved by the institutional review board for human research at Seoul National University Hospital (H-1007-049-323) and Ajou University Hospital (AJIRB-GN2-11-003). The written consent was acquired from the patients prior to conducting the study.

RESULTS

Clinical profiles of the study patients

Baseline demographics of study subjects are shown in Table 1. In total, 1,439 Korean subjects were included in this study and classified into two groups: 404 spontaneously recovered (SR) subjects and 1,035 chronic HBV carriers. Chronic HBV carriers were composed of 313 chronic hepatitis B, 305 liver cirrhosis and 417 HCCs (Table 1). In chronic carrier group, patients with more progressive status tended to be older and had higher male to female ratio and lower hepatitis Be antigen (HBeAg) positivity.

Table 1. Clinical profiles of the study patients

Clinical profiles	Spontaneously recovered	Chronic carrier		
		Hepatocellular carcinoma	Liver cirrhosis	Chronic hepatitis
No. of patients	404	417	305	313
Age (mean [range])	53.1 (28-81)	57.5 (25-82)	50.8 (22-90)	44.4 (18-79)
Sex (male/female)	272/132	279/48	231/74	238/75
HBeAg (positive rate)	0%	25.7%	29.7%	37.1%
HBeAb (positive rate)	37.7%	65.8%	50.2%	44.9%
HBsAg (positive rate)	0%	100%	100%	100%
HBsAb (positive rate)	100%	0%	0%	0%

Genotype and minor allele frequency of pre-miRNA 604 SNP (rs2368392) in Korean population

Fig. 1 shows schematic gene map and SNP. Nucleotide sequence of miR-604 on chromosome 10p11.23 (rs2368392) is marked with an arrow head (Fig. 1). The frequency in parentheses was based on the genotyping data. They were transcribed from chromosome 10 and their coordinate in human genome build 37 was 20834003, which had CC, CT, and TT genotypes. The genotype distributions of miR-604 rs2368392, in enrolled subjects, are as follows; CC genotype (46.4%), CT genotype (42.0%) and TT genotype (11.5%). The minor allele frequency of rs2368392 C > T was 0.326 and its heterozygosity was 0.439. The P value of deviation, from Hardy-Weinberg Equilibrium among spontaneously recovered group, was 0.229.

Association analysis of rs2368392 on pre-miRNA, miR-604 with risk of liver disease in Korean population

Table 2 shows the genotype distribution in the HCC and chronic HBV carrier, without HCC (chronic hepatitis or liver cirrhosis) groups.

First, we analyzed association of SNPs on pre-miRNA, miR-604 with risk of HBV persistence. Patients with T allele at pre-miRNA rs2368392 had a 1.19 odds of persistent HBV infection, and the rs2368392 C > T was found to be marginally associated with persistence of HBV infection (OR, 1.19; 95% CI, 1.00-1.42; P = 0.05 in a co-dominant model, and OR, 1.26; 95% CI, 1.00-

1.60; P = 0.05 in a dominant model) (Table 2).

Next, we performed an association analysis of rs2368392 polymorphisms, and the presence of HCC among patients with chronic HBV infection. There was a higher rate of the C allele in the HCC patients, compared to that of the chronic HBV infection without HCC patients. On the basis of unconditional logistic regression analysis, with adjustment for age and gender, rs2368392 T allele has protective effects on HCC occurrence (OR, 0.80; 95% CI, 0.65-0.98; P = 0.03 in a co-dominant model, and OR, 0.58; 95% CI, 0.38-0.91; P = 0.02 in a recessive model). Further, rs2368392 T allele also showed protective effect to the occurrence of HCC in liver cirrhosis group (OR, 0.80; 95% CI, 0.64-1.00; P = 0.05 in a co-dominant model, and OR, 0.53; 95% CI, 0.33-0.86; P = 0.009 in a recessive model).

However, we observed no statistically significant association between rs2368392 C > T SNP and the disease progression to cirrhosis or HBeAg clearance in patients with chronic HBV infection.

DISCUSSION

In this study, we analyzed SNP in the genomic region of miRNA, as candidate loci for HBV related disease progression and HCC susceptibility in the association studies. We examined whether the miR-604 genotype correlated with the HBV persistence and the risk for HCC in patients with HBV related chronic hepatitis or cirrhosis and spontaneously recovered individuals. We identified T allele in pre- miR-604 rs2368392 was associated with persistence of HBV infection and reduced HCC risk in patients with chronic HBV infection. Our findings provide genomic implication that miR-604 polymorphism may play a role in the development of liver cancer and in the natural course of HBV infection.

There are several investigations in regards to HCC and miRNA related genes. Recent studies imply considerable importance for both miR-155 and miR-21 in non-alcoholic steatohepatitis-associated HCC (13, 14), miR-96 in HBV associated HCC (15),

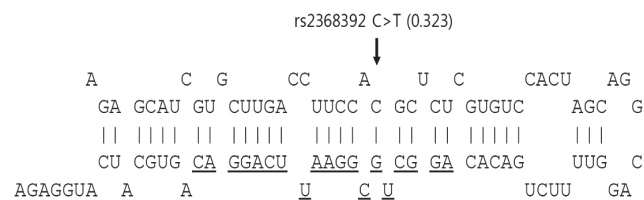


Fig. 1. Nucleotide sequence of miR-604 on chromosome 10p11.23. rs2368392 is marked with arrow head. The frequency in parentheses was based on the genotyping data (n = 1,439). The mature miRNA sequence is underlined.

Table 2. Association analysis of rs2368392 (C>T) on pre-miRNA, mir-604 with risk of liver disease in Korean (n=1,439)

Test group	Minor allele (T) frequency		Codominant		Dominant		Recessive	
	Case	Control	OR (95%CI)	P	OR (95%CI)	P	OR (95%CI)	P
HBV infection HBV (n = 1,035) vs. SR (n = 404)	0.336	0.298	1.19 (1.00-1.42)	0.05	1.26 (1.00-1.60)	0.05	1.25 (0.86-1.82)	0.25
HCC occurrence HCC (n = 417) vs. CH & LC (n = 618)	0.314	0.351	0.80 (0.65-0.98)	0.03	0.82 (0.62-1.08)	0.16	0.58 (0.38-0.91)	0.02
HCC occurrence on LC HCC (n = 417) vs. LC (n = 305)	0.314	0.362	0.80 (0.64-1.00)	0.05	0.86 (0.63-1.18)	0.36	0.53 (0.33-0.86)	0.009
LC occurrence on CH LC (n = 305) vs. CH (n = 313)	0.362	0.340	1.03 (0.81-1.31)	0.83	0.92 (0.66-1.29)	0.63	1.33 (0.82-2.17)	0.25
HBeAg clearance Chronic (n = 189) vs. Cleared (n = 428)	0.339	0.339	0.98 (0.76-1.27)	0.90	0.84 (0.59-1.19)	0.32	1.37 (0.83-2.27)	0.22

Minor allele frequencies and P values for logistic analyses of three alternative models (co-dominant, dominant, and recessive models) are shown. HBV, hepatitis B virus; HCC, hepatocellular carcinoma; CH, chronic hepatitis; LC, liver cirrhosis; SR, spontaneously recovered; HBeAg, hepatitis Be antigen.

and miR-126 in alcohol-induced HCC (16). MiR-143 induced by NF- κ B was found to be significantly up-regulated in HBV-related metastatic HCC (17). Moreover, marked up-regulation of miR-30d in metastatic HCC has been shown to enhance migration and invasion of HCC cells, and to promote intra-hepatic and distal pulmonary metastasis in an orthotopic mouse model (18).

Polymorphisms, which may affect miRNA mediated regulation of the cell, can be present not only in the 3' UTR of miRNA target gene, but also in the genes involved in miRNA biogenesis, and in processed miRNAs. Genetic variation, in miRNA genes and their biogenesis pathway, has the potential to affect the regulation of multiple cellular pathways in cancer development and susceptibility (5, 19). Polymorphisms in miRNA related genes also have been implicated in HCC susceptibility. A G > C polymorphism in miR-146a precursor (rs2910164) predicted HCC development (20), and male individuals with GG genotype were two fold more susceptible to HCC, compared with those with CC genotype. An insertion/deletion polymorphism at miRNA-122 binding site interleukin-1 alpha 3' UTR confers risk for HCC (21). Qi et al, reported that miR-196a-2 rs11614913 was associated with susceptibility to HBV-related HCC in Chinese male population (22).

In this case-control study for the Korean population, we found that rs2368392 T allele on pre-miRNA, miR-604, was a protective factor for the occurrence of HCC in patients with HBV related chronic liver disease. This study presents supporting evidence that miR-604 variants might be useful for identifying patients at high risk for progression to HBV-related HCC.

The natural history of HBV infection varies according to the age at which the infection was contracted (23). Although HBV infection age is important as a contributing factor to the natural course of HBV infection, it is unlikely to solely explain the HBV persistence, especially in the population whose transmission route is vertical infection, means almost all individuals had been exposed to the virus at the same age. In our study, the chronicity of HBV infection was higher in subjects with miR-604 T allele, compared with that of the subjects with C allele, which implies that the patients with miR-604 T allele may have a higher risk for HBV persistence, even though the statistical significance was marginal.

In addition to the association with viral resolution, SNP of miR-604 showed an association to the development of HCC in our work. Interestingly, T allele was associated with the persistence of HBV infection, but in contrast, it was a protective factor for the development of HCC. The involvement of the immune system in HCC development has been previously suggested and increased activity of the helper T cells, which promote inflammation, is associated with HCC (24). Furthermore, chronic inflammation has been implicated in the development of HCC (25, 26). Thus, polymorphisms of miR-604 may have opposite

roles in HBV eradication and HCC development in view of immune response. Unfortunately, functional mechanism and biological plausibility of miR-604 associated with the development of HCC/HBV clearance is not addressed by this study. The function of miR-604 in cancer has not been studied extensively and it remains to be defined whether miR-604 function to modulate host immunity to eradicate virus during the early stage of infection or function through other mechanisms. The role of miR-604 in HBV resolution and hepatocarcinogenesis should be clarified in the future.

In conclusion, the present investigation indicated that the pre-miR-604 rs2368392 polymorphism might confer genetic susceptibility to the occurrence of HCC in HBV related chronic liver disease, and HBV persistence after HBV infection. Such screening test may allow identification of individuals at increased risk of HCC for more intensive monitoring. Larger well-designed epidemiological studies, with ethnically diverse populations and functional evaluations, are warranted to confirm our findings.

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DISCLOSURE

We have nothing to declare in terms of conflicts of interest in this study

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