MicroRNA Gene Polymorphisms and Environmental Factors Increase Patient Susceptibility to Hepatocellular Carcinoma

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Abstract

Background: Micro RNAs (miRNAs) are small RNA fragments that naturally exist in the human body. Through various physiological mechanisms, miRNAs can generate different functions for regulating RNA protein levels and balancing abnormalities. Abnormal miRNA expression has been reported to be highly related to several diseases and cancers. Single-nucleotide polymorphisms (SNPs) in miRNAs have been reported to increase patient susceptibility and affect patient prognosis and survival. We adopted a case-control research design to verify the relationship between miRNAs and hepatocellular carcinoma.

Methodology/Principal Findings: A total of 525 subjects, including 377 controls and 188 hepatocellular carcinoma patients, were selected. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and real-time PCR were used to analyze miRNA146a (rs2910164), miRNA149 (rs2292832), miRNA196 (rs11614913), and miRNA499 (rs3746444) genetic polymorphisms between the control group and the case group. The results indicate that people who carry the rs3746444 CT or CC genotypes may have a significantly increased susceptibility to hepatocellular carcinoma (adjusted odds ratio [AOR] = 2.84, 95% confidence interval [CI] = 1.88–4.30). In addition, when combined with environmental risk factors, such as smoking and alcohol consumption, interaction effects were observed between gene polymorphisms and environmental factors (odds ratio [OR] = 4.69, 95% CI = 2.52–8.70; AOR = 3.38, 95% CI = 1.68–6.80).

Conclusions: These results suggest that a significant association exists between miRNA499 SNPs and hepatocellular carcinoma. Gene-environment interactions of miRNA499 polymorphisms, smoking, and alcohol consumption might alter hepatocellular carcinoma susceptibility.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide and the second leading cause of cancer-related death in Taiwan. In addition to common risk factors such as hepatitis B and hepatitis C, recent studies have shown that gene mutations may influence the inflammation mechanism that contributes to cell differentiation, proliferation, and apoptosis and connects several diseases and cancers [1–3]. Genotypes and mutations on hepatitis were associated to HCC, such as genotype C HBV increased the risk of cirrhosis and HCC, and genotype B HBV is associated with HCC in the patients under 50 years old [4]. Also, viral mutations on the promoter region and preS region are significantly increased the risk of HCC. The variances of genotype, mutations and viral load can be used as a prediction of HCC [5].

Micro RNAs (miRNAs) are small RNA fragments typically composed of only 20–22 nucleotides. MiRNAs can target specific mRNAs and negatively regulate their translational efficiency and stability to control the downstream cellular process including proliferation, differentiation, and survival [6,7]. According to the various functions of miRNAs, some studies have associated miRNAs with the immune system and inflammatory diseases [8,9]. Moreover, based on the functions that regulate cell differentiation, proliferation, and survival, several studies have identified a relationship between miRNAs and cancer [10–14].

Single-nucleotide polymorphisms (SNPs) are variations of DNA sequences that occur when nucleotides (A, T, C, or G) are changed

by at least 1% in certain populations. Previous epidemiologic studies have shown that genetic variations in miRNAs are associated with various diseases and cancers [15–18]. Several studies have provided evidence that SNPs in miRNAs may be related to patients' clinical prognosis [19–22], and high expression of miRNA causes superior survival. According to the various functions, low expression of miRNA can yield opposite results [23,24].

Based on the aforementioned reasons, we adopted a case-control research design and 4 pre-miRNA SNPs associated with cancer, namely, has-mir-146a (rs2910164), has-mir-149 (rs2292832), has-mir-196a2 (rs11614913), and has-mir-499 (rs3746444) [25–29]. We also considered the environmental risk factors for hepatocellular carcinoma such as smoking status and alcohol consumption. Furthermore, the clinical stage and laboratory status were combined to define the relationship between SNPs and patient susceptibility to hepatocellular carcinoma.

Materials and Methods

Subjects Selection

This study recruited 188 HCC patients at the Chung Shan Medical University Hospital, Taiwan. The diagnosis of HCC was made according to the criteria specified in the national guidelines for HCC. During the same study period, 337 ethnic group-matched individuals were enrolled as the controls that entered the physical examination at the same hospital. These control groups had neither self-reported history of cancer of any sites. As for cases and controls, exposure information, including tobacco use (smoker vs. non-smoker) and alcohol consumption (current heavy drinker, defined by CDC as consuming an average of more than 2 drinks per day vs. not current heavy drinker), were all obtained from questionnaires. We also collected the laboratory status such as α-Fetoprotein, AST, ALT and AST/ALT ratio for the further analysis. Before commencing the study, approval was obtained from the Institutional Review Board of Chung Shan Medical University Hospital and informed written consent was obtained from each individual.

DNA Extraction

We collected the whole blood samples from healthy controls and hepatocellular carcinoma patients with tubes containing EDTA, after centrifuged and stored at $-20°C$. The venous blood from each subject was drawn into Vacutainer tubes containing EDTA and stored at $4°C$. Genomic DNA was extracted by QIAamp DNA blood mini kits (Qiagen, Valencia, USA) according to the manufacture's instructions and the DNA was dissolved in TE buffer [10 mM Tris (PH 7.8), 1 mM EDTA] and then quantitated by a measurement of OD260. Final DNA preparation was stored at $-20°C$ and used as templates for the following experiments [30].

Polymerase Chain Reaction-restriction Fragment Length Polymorphism (PCR-RFLP)

The SNP of miRNAs gene rs2910164, rs11614913, and rs3746444 polymorphisms were determined by PCR-RFLP assay [31]. Amplify The has-mir-146a rs2910164 genotype were used the forward primer 5′- CATGGGTTGTGTCAGTGTCAGAG-CT-3′ and the reverse primer 5′- TGCCTTC TGTCTCCAGT-CTTCCAA-3′; the has-mir-196a2 rs 11614913 genotype were used forward primer 5′- CCCCTTCCCTTCTCCTCCAGATA-3′ and reverse primer 5′- CGAAAACCGACTGATGTAACTCCG-3′ and the has-mir-499 rs3746444 genotype were used forward primer 5′-CAAAGTCTTCACTTCCCTGCCA-3′ and reverse primer 5′-GATGTTTAACTCCTCTCCACGTGATC-3′. PCR was performed with total 10 μl volume with 100 ng DNA template,

Table 1. Demographic characteristics of controls and patients with hepatocellular carcinoma.

Variable	Control	Case	Total	p value
	N = 337 (%)	N = 188 (%)	N = 525 (%)	
Age				
<45	24 (7.12)	10 (5.05)	34 (6.48)	0.06
45–59	210 (40.06)	58 (30.85)	193 (36.76)	
>60	178 (52.82)	120 (63.83)	298 (56.76)	
Gender				
Women	85 (25.22)	52 (27.66)	137 (26.10)	0.54
Men	252 (74.78)	136 (72.34)	388 (73.90)	
Smoking status				
No	225 (66.77)	108 (57.45)	333 (63.43)	0.03*
Yes	112 (33.23)	80 (42.55)	192 (36.57)	
Alcohol intake				
No	201 (59.64)	120 (63.83)	321 (61.14)	0.35
Yes	136 (40.36)	68 (36.17)	204 (38.86)	
Hepatitis B virus[a]				
Negative	223 (86.77)	108 (57.45)	331 (74.38)	<0.001*
Positive	34 (13.23)	80 (42.55)	114 (25.62)	

*$p < 0.05$ considered statistically significant.
[a]80 frequency missing data in control group.
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1.0 μl of 10X PCR buffer (Invitrogen, Carslbad, CA, USA), 0.25 U of Taq DNA polymerase (Invitrogen), 0.2 mM dNTPs (Promega, Madison, WI, USA) and 200 nM of each primer (MDBio Inc. Taipei, Taiwan). The PCR conditions were 5 min at $94°C$ followed by 35 cycles of 30 sec at $94°C$, 30 sec at $58°C$ for hsa-mir-146a rs2910164, 30 sec at $63°C$ for has-mir-196a2 rs11614913, and 30 sec at $67°C$ for hsa-mir-499 rs3746444, and 1 min at $72°C$, and final step at $72°C$ for 10 min to allow a complete extension of all PCR fragments. PCR products of hsa-mir-146a rs2910164 gene polymorphism were subjected *SacI* to enzymatic digestion by incubation for 4 hr at $37°C$ and then submitted to electrophoresis in 2% agarose gels; *MspI* for hsa-mir-196a2 rs2910164 and *BclI* for hsa-mir-499 rs3746444.

Real-time PCR

MiRNA149 rs2292832 gene polymorphism was assessed using an ABI StepOne™ Real-Time PCR System (Applied Biosystems), SDS v3.0 software (Applied Biosystems), and the TaqMan assay. The final volume for each reaction mixture was 5 μL, containing 2.5 μL TaqMan Genotyping Master Mix, 0.125 μL TaqMan probes mix, and 10 ng genomic DNA. The reaction conditions included an initial denaturation step at $95°C$ for 10 min followed by 40 cycles at $95°C$ for 15 sec and $60°C$ for 1 min. For each assay, appropriate controls (nontemplate and known genotype) were included in each typing run to monitor reagent contamination and as a quality control. To validate results from real-time PCR, around 5% of assays were repeated and several cases of each genotype were confirmed by the DNA sequence analysis [32].

Statistical Analysis

Hardy–Weinberg equilibrium was assessed using a chi-square goodness-of-fit test for biallelic markers. The distributions of

Table 2. Distribution of miRNAs genotypes in healthy controls and hepatocellular carcinoma patients.

Gene	Control	Case	OR	AOR[a]
	N = 337 (%)	N = 188 (%)	(95%CI, *p* value)	(95%CI, *p* value)
miRNA146ars2910164				
CC	141 (41.84)	84 (44.68)	Reference	Reference
CG	146 (43.32)	82 (43.62)	0.94 (0.64–1.38, *p* = 0.763)	0.93 (0.63–1.37, *p* = 0.714)
GG	50 (14.84)	22 (11.70)	0.74 (0.42–1.31, *p* = 0.297)	0.72 (0.40–1.27, *p* = 0.253)
CG/GG	196 (58.16)	104 (55.32)	0.89 (0.62–1.28, *p* = 0.528)	0.83 (0.59–1.18, *p* = 0.300)
miRNA149 rs2292832				
TT	246 (73.00)	139 (73.94)	Reference	Reference
CT	64 (18.99)	36 (19.15)	0.99 (0.63–1.57, *p* = 0.984)	0.99 (0.62–1.58, *p* = 0.982)
CC	27 (8.01)	13 (6.91)	0.85 (0.43–1.71, *p* = 0.651)	0.82 (0.41–1.66, *p* = 0.579)
CT/CC	91 (27.00)	49 (26.06)	0.95 (0.64–1.43, *p* = 0.816)	0.94 (0.63–1.41, *p* = 0.767)
miRNA196 rs11614913				
TT	100 (29.67)	66 (35.11)	Reference	Reference
CT	167 (49.55)	81 (43.09)	0.74 (0.49–1.11, *p* = 0.140)	0.70 (0.46–1.06, *p* = 0.090)
CC	70 (20.77)	41 (21.81)	0.89 (0.54–1.46, *p* = 0.637)	0.93 (0.56–1.54, *p* = 0.785)
CT/CC	237 (70.33)	122 (64.89)	0.78 (0.53–1.14, *p* = 0.200)	0.90 (0.64–1.26, *p* = 0.544)
miRNA499 rs3746444				
TT	281 (83.38)	119 (63.30)	Reference	Reference
CT	55 (16.32)	60 (31.91)	2.58 (1.69–3.94, *p*<0.001)*	2.48 (1.62–3.81, *p*<0.001)*
CC	1 (0.30)	9 (4.79)	21.2 (2.66–169.55, *p* = 0.004)*	22.1 (2.73–178.40, *p* = 0.003)*
CT/CC	56 (16.62)	69 (36.70)	2.91 (1.93–4.40, *p*<0.001)*	2.91 (1.93–4.42, *p*<0.001)*

[a]AOR adjusted age, sex, smoking status and drinking status.
*p value <0.05.
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demographic characteristics and genotype frequencies between cases and controls in different genotypes were analyzed by Chi-square test, and Fisher's exact test were using at small sample size was present in some categories of variables. The Student's T-test was using to estimate laboratory status between the two groups. The odds ratios (ORs) and their 95% confidence intervals (CIs) of the association between genotype frequencies and hepatocellular carcinoma were estimated by multiple logistic regression models,

Table 3. Relationship of clinical TNM stages and miRNAs genotypes in liver cancer patients.

Variables	rs2910164			rs2292832			rs11614913			rs3746444		
	CC	CG or GG	*p* value	TT	CT or CC	*p* value	TT	CT or CC	*p* value	TT	CT or CC	*p* value
	N = 84 (%)	N = 104 (%)		N = 139 (%)	N = 49 (%)		N = 66 (%)	N = 122 (%)		N = 119 (%)	N = 69 (%)	
Clinical stage												
Stage I/II	49 (58.33)	66 (63.46)	0.471	85 (61.15)	30 (61.22)	0.992	40 (60.61)	75 (61.48)	0.911	73 (61.34)	42 (60.87)	0.947
Stage III/IV	35 (41.67)	38 (36.54)		54 (38.85)	19 (38.78)		26 (39.39)	47 (38.52)		46 (38.66)	27 (39.13)	
Tumor size												
T1+ T2	50 (59.52)	67 (64.42)	0.496	88 (63.31)	29 (59.18)	0.615	39 (59.09)	78 (63.93)	0.510	75 (63.03)	42 (60.87)	0.775
T3+ T4	34 (40.48)	37 (35.58)		51 (36.69)	20 (40.82)		27 (40.91)	44 (36.07)		44 (36.97)	27 (39.13)	
Lymph node metastasis												
Negative	79 (94.05)	100 (96.15)	0.514[a]	131 (94.24)	48 (97.96)	0.294[a]	62 (93.94)	117 (95.90)	0.554[a]	112 (94.12)	67 (97.10)	0.353[a]
Positive	5 (5.95)	4 (3.85)		8 (5.76)	1 (2.04)		4 (6.06)	5 (4.10)		7 (5.88)	2 (2.90)	
Distant metastasis												
Negative	78 (92.86)	99 (95.19)	0.493	133 (95.68)	44 (89.80)	0.136	62 (93.94)	115 (94.26)	0.922[a]	110 (92.44)	67 (97.10)	0.194[a]
Positive	6 (7.14)	5 (4.81)		6 (4.32)	5 (10.20)		4 (6.06)	7 (5.74)		9 (7.56)	2 (2.90)	

[a]Use Fisher's exact test.
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Table 4. Relationship of clinical statuses and miRNAs genotypes in liver cancer patients.

Variables	rs2910164			rs2292832			rs11614913			rs3746444		
	CC	CG or GG	*p* value	TT	CT or CC	*p* value	TT	CT or CC	*p* value	TT	CT or CC	*p* value
	N = 84 (%)	N = 104 (%)		N = 139 (%)	N = 49 (%)		N = 66 (%)	N = 122 (%)		N = 119 (%)	N = 69 (%)	
HBsAg												
Negative	52 (61.90)	57 (54.81)	0.322	82 (58.99)	27 (55.10)	0.633	33 (50.00)	76 (62.30)	0.102	66 (55.46)	43 (62.32)	0.357
Positive	32 (38.10)	47 (45.19)		57 (41.01)	22 (44.90)		33 (50.00)	46 (37.70)		53 (44.54)	26 (37.68)	
Anti-HCV												
Negative	44 (52.38)	53 (50.96)	0.841	69 (49.64)	28 (57.14)	0.367	34 (51.52)	63 (51.64)	0.983	67 (56.30)	30 (43.48)	0.079
Positive	40 (47.62)	51 (49.04)		70 (50.36)	21 (42.86)		32 (48.48)	59 (48.36)		52 (43.70)	39 (56.52)	
Child-Pugh grade												
A	62 (73.81)	75 (72.12)	0.796	103 (74.10)	34 (69.39)	0.526	46 (69.70)	91 (74.59)	0.475	85 (71.43)	52 (75.36)	0.551
B or C	22 (26.19)	29 (27.88)		36 (25.90)	15 (30.61)		20 (30.30)	31 (25.41)		34 (28.57)	17 (24.64)	
Liver Cirrhosis												
Negative	21 (25.00)	29 (27.88)	0.654	41 (29.50)	9 (18.37)	0.129	13 (19.70)	37 (30.33)	0.113	30 (25.21)	20 (28.99)	0.572
Positive	63 (75.00)	75 (72.12)		98 (70.50)	40 (81.63)		53 (80.30)	85 (69.67)		89 (74.79)	49 (71.01)	

[a]Use Fisher's exact test.
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also controlling for covariates. The *p* value of less than 0.05 was considered significant. The data were analyzed on SPSS 12.0 statistical software.

Results

Study Population

The demographic characteristics of the 2 groups, including age, sex, smoking status, and alcohol consumption, are shown in Table 1. A significant difference in smoking status was observed between the 2 groups ($p = 0.03$).

MiRNA Gene Polymorphisms

The distribution of miRNA gene polymorphisms is described in Table 2. In our recruited control group, the frequencies of miRNA146a rs2910164 (χ^2 value: 1.92), miRNA196 rs11614913 (χ^2 value: 0.0005), and miRNA499 rs3746444 (χ^2 value: 0.98) were in Hardy-Weinberg equilibrium, respectively, except for

Table 5. Relationship of HCC laboratory status and miRNAs genotypes in liver cancer patients.

Variables	α-Fetoprotein	AST	ALT	AST/ALT
	(ng/ml)	(IU/L)	(IU/L)	ratio
miRNA146a rs2910164				
CC	4211.1±18853	192.93±363.25	122.52±171.11	1.73±1.44
CG/GG	4459.9±16050	196.55±389.84	196.84±362.98	1.25±0.70
p value	0.922	0.948	0.086	0.003*
miRNA149 rs2292832				
TT	3584.9±16267	198.77±401.56	170.74±320.38	1.42±1.04
CT/CC	6515.6±20006	184.02±300.92	143.48±207.47	1.59±1.33
p value	0.309	0.815	0.579	0.359
miRNA196 rs11614913				
TT	3599.4±14256	199.35±416.04	173.52±332.77	1.42±0.74
CT/CC	4754.1±18801	192.54±356.22	158.29±273.48	1.49±1.29
p value	0.664	0.906	0.736	0.705
miRNA499 rs3746444				
TT	2871.7±11986	214.91±434,71	167.92±333.43	1.54±1.24
CT/CC	6896.2±23757	160.48±247.88	156.25±214.63	1.34±0.87
p value	0.125	0.342	0.794	0.254

*p value <0.05.
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Table 6. Association of miRNA genotype and smoking status.

Variable	Control	Case	OR	AOR[a]
	N = 337 (%)	N = 188 (%)	(95%CI, *p* value)	(95%CI, *p* value)
miRNA146a rs2910164				
CC and non-smoker	94 (27.89)	50 (26.60)	Reference	Reference
CG or GG and non-smoker	131 (38.87)	58 (30.85)	0.83 (0.53–1.32, *p* = 0.436)	0.80 (0.50–1.31, *p* = 0.376)
CC and smoker	47 (13.95)	34 (18.09)	1.36 (0.78–2.38, *p* = 0.280)	1.37 (0.78–2.47, *p* = 0.293)
CG or GG and smoker	65 (19.29)	46 (24.47)	1.33 (0.80–2.22, *p* = 0.273)	1.36 (0.78–2.36, *p* = 0.278)
Test for interaction	χ^2 = 5.102 (3 d.f.), *p* = 0.164			
miRNA149 rs2292832				
TT and non-smoker	164 (48.66)	50 (43.09)	Reference	Reference
CT or CC and non-smoker	61 (18.10)	27 (14.36)	0.90 (0.53–1.52, *p* = 0.682)	0.85 (0.49–1.47, *p* = 0.550)
CT or CC and smoker	82 (24.33)	58 (30.85)	1.43 (0.93–2.20, *p* = 0.101)	1.66 (1.05–2.64, *p* = 0.003)*
CT or CC and smoker	30 (8.90)	114 (11.70)	1.49 (0.81–2.74, *p* = 0.205)	1.88 (0.99–3.59, *p* = 0.054)
Test for interaction	χ^2 = 4.689 (3 d.f.), *p* = 0.1960			
miRNA196 rs11614913				
TT and non-smoker	74 (21.96)	34 (18.09)	Reference	Reference
CT or CC and non-smoker	151 (44.81)	74 (39.36)	1.07 (0.65–1.75, *p* = 0.797)	0.99 (0.59–1.66, *p* = 0.962)
CT or CC and smoker	26 (7.72)	32 (17.02)	2.68 (1.39–5.17, *p* = 0.003)*	2.69 (1.34–5.41, *p* = 0.005)*
CT or CC and smoker	86 (25.52)	48 (25.53)	1.22 (0.71–2.08, *p* = 0.479)	1.21 (0.68–2.13, *p* = 0.516)
Test for interaction	χ^2 = 11.175 (3 d.f.), *p* = 0.010*			
miRNA499 rs3746444				
TT and non-smoker	188 (55.79)	74 (39.36)	Reference	Reference
CT or CC and non-smoker	37 (10.98)	34 (18.09)	2.34 (1.36–4.00, *p* = 0.002)*	2.41 (1.36–4.26, *p* = 0.002)*
CT or CC and smoker	93 (27.60)	45 (23.94)	1.23 (0.79–1.92, *p* = 0.364)	1.37 (0.85–2.20, *p* = 0.192)
CT or CC and smoker	19 (5.64)	35 (18.62)	4.69 (2.52–8.70, *p*<0.001)*	5.64 (2.87–11.06, *p*<0.001)*
Test for interaction	χ^2 = 31.409 (3 d.f.), *p*<0.001*			

[a]AOR adjusted age, gender and alcohol intake.
*p value <0.05.
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miRNA149 rs2292832 (χ^2 value: 59.86). By using a multiple logistic regression model to estimate the adjusted odds ratio (AOR), we observed a significantly higher risk for people who carried the CT (AOR = 2.52, 95% confidence interval [CI] = 1.63–3.86) or CC (AOR = 20.7, 95% CI = 2.60–165.90) genotypes of miRNA499 (rs3746444). Because of the insufficient size of the CC group, the CT and CC genotype samples were combined, producing similar results (AOR = 2.84, 95% CI = 1.88–4.30). In addition, no statistical significance was observed in the other 3 gene polymorphisms (rs2910164, rs2292832, and rs11614913). Similarly, a significantly higher risk for people who carried the CT (AOR = 6.14, 95% confidence interval [CI] = 1.32–28.56) or CC (AOR = 4.75, 95% CI = 1.32–17.01) genotypes of miRNA499 (rs3746444) was observed in the HBV-infection group (Table S1 in File S1).

Gene Polymorphisms with Clinicopathologic TNM Staging

The distribution of clinical status and miRNA polymorphisms in HCC patients were estimated to clarify the role of miRNA polymorphisms in the clinicopathologic state of HCC patients. No significant association was found between the levels of these HCC clinical pathological markers as well as presence of HBV or HCV, liver cirrhosis and genotypes for any of the RNA SNPs in HCC patients (Table 3 and Table 4). However, the trend of TC+CC

genotype of miRNA rs2292832 for distant metastasis risk was higher in the male participants (Table S2 in File S1) while that of female participants was not (Table S3 in File S1).

Gene Polymorphisms with Laboratory Status

We associated laboratory status with gene polymorphisms, including α-fetoprotein (ng/mL), AST (IU/L), ALT (IU/L), and AST/ALT ratios, as shown in Table 5. We observed a significantly lower ratio in the patients who carried rs2910164 CG or GG genotypes (*p* = 0.003).

Gene Polymorphism with Environmental Risk Factors

Furthermore, we considered the environmental risk factors of hepatocellular carcinoma such as smoking and alcohol consumption. The risk factors were then combined with miRNA gene polymorphisms and examined to determine whether interactions existed between the 2 factors. The relationship between genotype and smoking status indicated that people who carried rs3746444 CT or CC genotypes and non-smoking habits had an increased odds ratio (OR = 2.34, 95% CI = 1.36–4.00), and those with CT or CC genotypes and smoking habits had the highest odds ratio (OR = 4.69, 95% CI = 2.52–8.70) (Table 6). The result for patients who carried the re3746444 CT or CC genotypes was similar to that observed for patients who consumed alcohol (AOR = 3.44, 95% CI = 1.69–7.00) (Table 7).

Table 7. Association of miRNA genotype and alcohol intake status.

Variable	Control	Case	OR	AOR[a]
	N = 337 (%)	N = 188 (%)	(95%CI, *p* value)	(95%CI, *p* value)
miRNA146a rs2910164				
CC and non-alcohol intake	88 (26.11)	53 (28.19)	Reference	Reference
CG or GG and non-alcohol intake	113 (33.53)	67 (35.64)	0.98 (0.62–1.55, *p* = 0.946)	0.94 (0.59–1.49, *p* = 0.792)
CG or GG and consumer	53 (15.73)	31 (16.49)	0.97 (0.56–1.70, *p* = 0.918)	0.87 (0.48–1.57, *p* = 0.651)
CG or GG and alcohol intake	83 (24.63)	37 (19.68)	0.74 (0.44–1.24, *p* = 0.253)	0.64 (0.37–1.10, *p* = 0.110)
Test for interaction	χ^2 = 1.687 (3 d.f.) *p* = 0.640			
miRNA149 rs2292832				
TT and non-alcohol intake	140 (41.54)	91 (48.40)	Reference	Reference
CT or CC and non-alcohol intake	61 (18.10)	29 (15.43)	0.73 (0.44–1.22, *p* = 0.234)	0.74 (0.44–1.26, *p* = 0.269)
CT or CC and consumer	106 (31.45)	48 (25.53)	0.70 (0.45–1.07, *p* = 0.100)	0.61 (0.38–0.97, *p* = 0.036)
CT or CC and alcohol intake	30 (8.90)	20 (10.64)	1.03 (0.55–1.92, *p* = 0.937)	0.98 (0.51–1.89, *p* = 0.955)
Test for interaction	χ^2 = 3.620 (3 d.f.) *p* = 0.306			
miRNA196 rs11614913				
TT and non-alcohol intake	64 (18.99)	38 (20.21)	Reference	Reference
CT or CC and non-alcohol intake	137 (40.65)	82 (43.62)	1.01 (0.62–1.64, *p* = 0.974)	0.95 (0.58–1.57, *p* = 0.854)
CT or CC and consumer	36 (10.68)	28 (14.89)	1.31 (0.69–2.48, *p* = 0.406)	1.05 (0.53–2.10, *p* = 0.884)
CT or CC and alcohol intake	100 (29.67)	40 (21.28)	0.67 (0.39–1.16, *p* = 0.154)	0.53 (0.29–0.96, *p* = 0.036)*
Test for interaction	χ^2 = 5.293 (3 d.f.) *p* = 0.152			
miRNA499 rs3746444				
TT and non-alcohol intake	163 (48.37)	78 (41.49)	Reference	Reference
CT or CC and non-alcohol intake	38 (11.28)	42 (22.34)	2.31 (1.38–3.87, *p* = 0.001)*	2.43 (1.43–4.12, *p* = 0.001)*
CT or CC and consumer	118 (35.01)	41 (21.81)	0.73 (0.47–1.13, *p* = 0.160)	0.66 (0.41–1.06, *p* = 0.085)
CT or CC and alcohol intake	18 (5.34)	27 (14.36)	3.14 (1.63–6.03, *p* < 0.001)*	3.44 (1.69–7.00, *p* < 0.001)*
Test for interaction	χ^2 = 29.345 (3 d.f.) *p* < 0.001*			

[a]AOR adjusted age, gender and smoking status.
p value < 0.05.
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Discussion

The results of this investigation indicate that miRNA499 rs3746444 is highly associated with hepatocellular carcinoma. Regarding genotype distribution and environmental risk factors, such as smoking and alcohol consumption, miRNA499 plays a crucial role in hepatocellular carcinoma.

MiRNA499 rs3746444 has been discussed in relation to numerous cancers. A meta-analysis of 7188 patients with several cancers compared with 8548 control participants reported that a significant association exists between miR499 rs3746444 polymorphisms and an increased risk of cancer [33,34]. MiRNA499 rs3746444 has been reported to increase susceptibility to hepatocellular carcinoma in the Chinese population [35]. The results obtained after increasing the sample size were similar. Several studies of Asian populations have reported the same finding; that is, miRNA499 is associated with cancer [36,37]. However, in Caucasian population, miRNA499 has not been associated with cancer [38]. Our findings may through the racial difference and it's specifically direct against to Asians. Furthermore, the basic laboratory study also demonstrated that miRNA499 may bind to and reduce the expression of c-MET mRNA, which promotes cell apoptosis and inhibits cell proliferation [39].

Previous studies have shown that miRNA146 can be used as a biomarker and therapeutic target for peripartum cardiomyopathy [40], and may negatively regulate the activity of bone marrow stem cells [41]. Other studies have linked miRNA146 to inflammatory immune system responses or T-cell activity [42,43]. Compared to the laboratory status, we observed a significant difference in the AST/ALT ratio of patients who carried CG or GG genotypes compared with those who carried the CC genotype, which may related to the liver damage caused by alcohol. Further, serum liver enzymes were associated with long-term mortality, especially the populations with low ALT and AST levels [44].

A slight difference exists in distant tumor metastasis caused by miRNA149 rs2292832 gene polymorphisms (Table S2 in File S1). Recent studies have shown that miRNA149 gene polymorphisms are associated with the prognosis for patients diagnosed with head and neck carcinoma [45]. Øster et al., also found that miRNA149 can regulate SPRX2 expression and CpG promotes hypomethylation in colorectal cancer [46]. Several in vivo studies have associated miRNA149 with the p53 gene and reported that miRNA149 may serve as an oncogenic regulator of p53 expression [47]. We observed a slight difference in distant tumor metastasis caused by miRNA149; however, a larger sample size and additional laboratory experiments are required to verify this finding.

In this study, miRNA196 exhibited a slight association with hepatocellular carcinoma after patients' alcohol consumption was considered. However, the test results for an interaction between gene polymorphisms and cancer risk factors were not statistically significant ($p = 0.152$). Previous studies have shown that miRNA196 is associated with transcription factors and affects cancer development and progression [48,49]. In addition, increased miRNA196 expression improves the survival and prognosis for patients diagnosed with leukemia [50]. Nonetheless, the results obtained in this research do not provide sufficient evidence of a relationship between miR196 and hepatocellular carcinoma. Furthermore, after considering environmental risk factors, such as smoking and alcohol consumption, we combined the gene polymorphisms and analysis results of the interactions caused by the 2 risk factors. Considered with the viral factors, carried miR34b/c rs4938723 CC was significantly associated with HCC. And with miR196a rs11614913 was not associated with HCC, but carried CC genotype significantly enhanced the influences caused by rs4938723 in women population [51]. For patients in the smoking and alcohol consumption groups, mirRNA499 can cause interactions ($p < 0.001$) and increase the OR for developing hepatocellular carcinoma. This may be because the various functions of miRNAs not only influence tumor differentiation and proliferation, but also promote inflammation and apoptosis.

Sample size was the greatest limitation in our study. The sample comprised a fewer number of female participants than male participants, which may be due to differences in lifestyle factors, such as smoking status and alcohol consumption. Substantial exposure to the environmental risk factors can easily result in underestimation of gene affections in subsequent analyses. We used a regression model to reduce the effect of potential confounding factors, but estimating the genetic effects under environmental affections remained difficult.

In conclusion, we observed a strong relationship between miRNA499 gene polymorphisms and hepatocellular carcinoma. People who carry miRNA499 rs3746444 CT or CC genotypes may have a high susceptibility to hepatocellular carcinoma. In addition, we observed a strong interaction and increased odds ratio for hepatocellular carcinoma development between miRNA499 gene polymorphisms and smoking and alcohol consumption. Although we observed evidence that miRNA499 rs3746444 is related to hepatocellular carcinoma susceptibility, additional laboratory experiments and functional assays are required to confirm the effects of miRNA499 rs3746444 gene polymorphisms on hepatocellular carcinoma.

Supporting Information

File S1 Distribution of miRNAs genotypes in liver cancer patients. Table S1. Distribution of miRNAs genotypes with Hepatitis B in healthy controls and liver cancer patients. Table S2. Relationship of clinical TNM stages and miRNAs genotypes in male liver cancer patients. Table S3. Relationship of clinical TNM stages and miRNAs genotypes in female liver cancer patients.
(DOC)

Author Contributions

Conceived and designed the experiments: YHC WHK. Performed the experiments: SFY MJH. Analyzed the data: HLC YSL CCY. Contributed reagents/materials/analysis tools: SFY WHK. Wrote the paper: YHC WHK.

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