Identification of *miR-423* and *miR-499* Polymorphisms on Affecting the Risk of Hepatocellular Carcinoma in a Large-Scale Population

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Aims: MicroRNAs (miRNAs) regulate gene expression and act as tumor suppressors or enhancers in oncogenesis. Single-nucleotide polymorphisms (SNPs) in miRNAs could alter the processing or actions of mature miRNA. So far, the association of *miR-423* rs6505162 with cancers has not been explored, while the association of *miR-499* rs3746444 was only reported in small-sized samples of different types of populations. **Methods:** To evaluate the association of *miR-499* rs3746444 and *miR-423* rs6505162 with hepatocellular carcinoma (HCC), we performed a large-scale case-control study of 984 patients with HCC and 991 cancer-free controls. **Results:** The risk of HCC was significantly higher with *miR-499* rs3746444 TC+CC genotypes compared with those with the TT genotype (odds ratio [OR]=1.372, 95% confidence intervals [CI]=1.099–1.713, $p=0.005$), as was the risk of hepatitis B virus-related HCC (OR=1.437, 95% CI=1.128–1.831, $p=0.003$). Moreover, subjects with the TC+CC genotypes were more vulnerable to advanced HCC with larger tumor size ($\chi^2=13.014$, $p=0.001$) and/or higher total bilirubin ($p=0.004$), which suggested that a TT genotype or T allele might serve as a protective factor. *miR-423* rs6505162 had no effect on the risk of HCC. **Conclusions:** *miR-499* rs3746444 may contribute to the risk and prognosis of HCC, indicating that this SNP could be developed as a biomarker for HCC prediction.

Introduction

LIVER CANCER IS THE fifth common neoplasm worldwide and the third common cause of cancer mortality, with an annual mortality of about 700,000 persons globally. Hepatocellular carcinoma (HCC) accounts for between 85% and 90% of primary liver cancers and is predominant in developing countries (El-Serag and Rudolph, 2007). Since it is often diagnosed at an advanced stage, HCC generally has a poor prognosis. To date, surgical resection and liver transplantation are the best curative options to treat liver cancer. However, according to the liver function and tumor status, currently only 5–15% of HCC patients are eligible for surgical intervention (Blum, 2005). Meanwhile, frequent tumor metastasis and tumor recurrence after surgical intervention often result in poor outcomes in HCC patients (Lu *et al.*, 2009). Therefore, identification of effective diagnostic and prognostic markers of HCC is of crucial significance.

MicroRNAs (miRNAs) are small single-stranded non-coding RNAs of about 22 nucleotides, and are master regulators of gene expression and control many biological pathways such as cell growth, differentiation, and apoptosis

(Bartel, 2004, 2009). Recently, many studies have focused on miRNA expression profiling in liver cancer, which shows that any slight alterations in miRNA expression may influence cancer formation (Calin and Croce, 2006). Single-nucleotide polymorphisms (SNPs) within miRNAs could affect transcription, processing, or target recognition and result in malignant diseases (Pastinen *et al.*, 2006; Buonocore *et al.*, 2010). In addition, most of the known miRNAs have a large number of potential targets in mRNAs; minor variations in miRNA can influence the expression of a large number of proteins. Several researchers have reported that *miR-499* rs3746444, *miR-196a2* rs11614913, *miR-34b/c* rs4938723, and so on may be correlated to susceptibility for HCC (Lafferty-Whyte *et al.*, 2009; Wang *et al.*, 2011; Xu *et al.*, 2011; Guo *et al.*, 2012; Fan *et al.*, 2013; Zhang *et al.*, 2013).

miR-423 and *miR-499* are mapped on chromosome 17q and 20q, respectively. Both of them participate in oncogenesis, and their SNPs, namely rs6505162 and rs3746444, have shown influence on susceptibility to a range of cancers, such as colorectal cancer (Xing *et al.*, 2012), breast cancer (Al-shatwi *et al.*, 2012; Smith *et al.*, 2012), head and neck cancer (Liu *et al.*, 2010), esophageal cancer (Ye *et al.*, 2008), and

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ovarian cancer (Kontorovich *et al.*, 2010). Especially, *miR-423* could distinguish hepatitis B virus (HBV)-related HCC from healthy controls, serving as a diagnostic biomarker for HBV-related HCC (Li *et al.*, 2010). rs6505162 is in a stem-loop structure of *miR-423* and has the potential to change the expression or process of *miR-423* (Duan *et al.*, 2009); however, the relationship between *miR-423* and genetic predisposition to HCC in any kind of ethnic group is unclear. Although a couple of association studies of *miR-499* rs374644 risk of HCC have been performed, conflicting results have been reported due to small sample size and non-comparable ethnic groups.

Therefore, this study in a large-scale sample aims at verifying whether rs6505162 and rs3746444 affect the risk of HCC.

Materials and Methods

Study population

In our study, 984 HCC cases and 991 controls were enrolled from an unrelated East Chinese population sample collected from Huashan Hospital, Eastern Hepatobiliary Surgery Hospital, and Taizhou Longitudinal Study as previously described (Zhang *et al.*, 2013). In brief, all HCC patients were confirmed by pathologic or imaging certification, and the controls had no history of cancer or other serious diseases. The basic features of all enrolled subjects included age, gender, family history, smoking status, and alcohol status. For HCC patients, clinicopathologic characteristics, such as serum alpha-fetoprotein (AFP) levels, hepatitis B surface antigen (HBsAg) status, HBV-DNA titer, alanine aminotransferase (ALT), aspartate transaminase (AST), total bilirubin (TB), tumor number/size, and tumor grade, were also revealed. All the enlisted cases and controls were approved by the local ethics review board.

DNA extraction

A 5 mL sample of peripheral blood was collected from each subject. Genomic DNA was extracted from the blood sample by using the AxyPrep™ Blood Genomic DNA Miniprep Kit (Axygen Biosciences). For the sake of the accuracy of subsequent experiments, all DNA samples were subjected to electrophoresis and concentration determination. Sequenom MassARRAY with clear strips in electrophoresis and above 10 ng/μL was performed to genotype these samples.

Genotyping

Amplification primers and extension primer of the SNPs, which were obtained by MassARRAY Assay Design, are listed in Table 1. Genotyping of the SNPs was performed by Sequenom MassARRAY technology, which was carried out by a MassARRAY Analyzer Compact system (Sequenom Corporation) and analyzed by TYPER 4.0.

Statistical analysis

The computer software Statistical Package for Social Sciences (SPSS, version 13.0) and Excel were used to conduct all data analysis. Hardy–Weinberg equilibrium was evaluated by the comparison between the observed genotype frequencies

TABLE 1. THE SELECTED TWO SINGLE-NUCLEOTIDE POLYMORPHISMS SITES IN MICRORNAs

SNP ID	Substitutes	miRNA	SNP location	Chromosome	start-stop site	Amplification primers	Extension primer
rs6505162	A/C	hsa-mir-423	28444183 (stem-loop structure)	chr17:28444097-28444190	ACGTTGGATGTTTCCAAAAGCTCGGTCTG ACGTTGGATGCAAGCGGGAGAACTCAAG	CTCAGTCTTGCTTCTCTA	
rs3746444	C/T	hsa-mir-499	33578251 (mat)	chr20:33578179-33578300	ACGTTGGATGGGCTGTAAAGACTTGCAAGT ACGTTGGATGGGAAGCAGCACAGACTTG	CCTCTCCACGTGAAC	

miRNA, microRNA; SNP, single-nucleotide polymorphisms.

TABLE 2. GENERAL CHARACTERISTICS IN HEPATOCELLULAR CARCINOMA PATIENTS AND CONTROLS

	Cases (n=984)	Controls (n=991)	p-Value
	n (%) or mean $\pm$ SD	n (%) or mean $\pm$ SD	
Age (years)	54.67 $\pm$ 11.24	59.6 $\pm$ 11.59	<0.001
Gender			
Male	810 (82.3)	718 (72.5)	<0.001
Female	174 (17.7)	273 (27.5)	
Smoking status			
Never	657 (67.7)	526 (53.1)	<0.001
Ever	314 (32.3)	465 (46.9)	
Drinking status			
Never	726 (74.6)	734 (74.1)	0.781
Ever	247 (25.4)	257 (25.9)	
HBsAg (n=938)			
Negative	178 (19.0)		
Positive	760 (81.0)		
Tumor size (n=536)			
<5 cm	217 (40.5)		
$\geq$ 5 cm	319 (59.5)		
Tumor number (n=535)			
Single	472 (88.2)		
Multiple	63 (11.8)		
Tumor grade (n=382)			
I–II	84 (22.0)		
III–IV	298 (78.0)		
Serum level of tumor markers			
ALT (U/L, in 992 subjects)	58.61 $\pm$ 86.59		
AST (U/L, in 988 subjects)	62.36 $\pm$ 81.47		
AFP			
<20 μ g/L	359 (37.3)		
$\geq$ 20 μ g/L	603 (62.7)		
(μ g/L, in 396 subjects) ^a	124.01 $\pm$ 287.04 (0.6–1210)		
HBV-DNA (IU/mL, in 444 subjects)	1.762E06 $\pm$ 5.452E06 (1000–6.9E07)		

^aQuantity data available in 396 subjects.

AFP, alpha-fetoprotein; ALT, alanine aminotransferase; AST, aspartate transaminase; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus.

and the expected ones. Binary logistic regression was used to appraise the differences in the genotypes or allele frequencies between the cases and controls, by which odds ratios (ORs) and 95% confidence intervals (CI) were calculated, so as to estimate the relative risk of these SNPs adjusted for smoking, alcohol, and other confounding factors. Pearson's χ^2 tests were performed to detect the association between clinicopathologic characteristics and miRNA genotypic/allelic frequencies in HCC patients. For the other quantitative variables that had heterogeneity of variance or non-normal distributions, analysis of variance or nonparametric tests were applied. All statistical tests were two sided. A *p*-value <0.05 was considered significantly different.

Results

Characteristics of the case-control sample

As shown in Table 2, this study included 984 HCC patients and 991 healthy subjects. Among all cases, 760 HBsAg-positive subjects were defined as HBV-related HCC. For HCC patients, further clinicopathologic characteristics were also cataloged, such as AFP, ALT, AST, and HBV-DNA. It was shown that age, gender, and smoking status had significant

differences between the case and the control groups, while alcohol consumption had no discrepancy between the two groups.

miR-423 and miR-499 polymorphisms and the risk of HCC

Since the genotype distributions of the SNPs obeyed Hardy–Weinberg equilibrium in both groups, association between the genotype/allele and the risk of HCC or HBV-related HCC was subsequently analyzed by binary logistic regression.

As shown in Table 3, TC and CC genotype in patients with miR-499 rs3746444 increased the risk of HCC/HBV-related HCC in contrast with wild genotype TT (HCC: OR = 1.372, 95% CI = 1.099–1.713, *p* = 0.005; HBV-related HCC: OR = 1.437, 95% CI = 1.128–1.831, *p* = 0.003, respectively). The C allele seemed to be a risk factor of HCC/HBV-related HCC (HCC: OR = 1.236, 95% CI = 1.013–1.507, *p* = 0.037; HBV-related HCC: OR = 1.263, 95% CI = 1.017–1.569, *p* = 0.035, respectively). Further, a gender stratification study (Table 4) showed that subjects with miR-499 rs3746444 TC + CC genotype or C allele had a considerably higher risk of HCC in men (TC + CC genotype: OR = 1.538, 95% CI = 1.194–1.981, *p* = 0.001; C

TABLE 3. ASSOCIATION BETWEEN GENOTYPES/ALLELES OF TWO POLYMORPHISMS AND THE RISK OF HEPATOCELLULAR CARCINOMA/HEPATITIS B VIRUS-RELATED HEPATOCELLULAR CARCINOMA

Genotypes	Controls		HCC patients		HCC patients with HBV		
	n (%)	n (%)	OR (95% CI) ^a	p-Value ^a	n (%)	OR (95% CI) ^a	p-Value ^a
<i>miR-499</i> rs3746444	<i>n</i> = 969	<i>n</i> = 984			<i>n</i> = 760		
TT	765 (79.0)	724 (73.6)	1.000		558 (73.4)	1.000	
TC	179 (18.4)	241 (24.5)	1.458 (1.157–1.836)	0.001 ^b	189 (24.9)	1.547 (1.203–1.990)	0.001 ^b
CC	25 (2.6)	19 (1.9)	0.759 (0.396–1.454)	0.405	13 (1.7)	0.685 (0.328–1.430)	0.313
Dominant model (TT vs. TC + CC)			1.372 (1.099–1.713)	0.005 ^b		1.437 (1.128–1.831)	0.003 ^b
Recessive model (TT + TC vs. CC)			1.433 (0.750–2.741)	0.276		0.622 (0.299–1.295)	0.204
T	1709 (88.2)	1689 (85.8)	1.000		1305 (85.9)	1.000	
C	229 (11.8)	279 (14.2)	1.236 (1.013–1.507)	0.037 ^b	215 (14.1)	1.263 (1.017–1.569)	0.035 ^b
<i>miR-423</i> rs6505162	<i>n</i> = 991	<i>n</i> = 984			<i>n</i> = 763		
CC	652 (65.8)	643 (65.3)	1.000		491 (64.4)	1.000	
CA	297 (30.0)	313 (31.8)	0.996 (0.812–1.222)	0.967	248 (32.5)	0.951 (0.761–1.189)	0.661
AA	42 (4.2)	30 (2.9)	0.668 (0.391–1.139)	0.138	24 (3.1)	0.725 (0.405–1.298)	0.279
Dominant model (CC vs. CA + AA)			1.038 (0.852–1.264)	0.714		1.017 (0.820–1.261)	0.878
Recessive model (CC + CA vs. AA)			0.669 (0.399–1.122)	0.128		0.750 (0.427–1.316)	0.316
C	1601 (80.8)	1599 (82.3)	1.000		1228 (80.6)	1.000	
A	381 (19.2)	343 (17.7)	0.935 (0.790–1.107)	0.438	296 (19.4)	0.987 (0.821–1.186)	0.887

^aOR and *p*-value were obtained after adjusting for age, gender, smoking status, and wine status.^b*p*-Value was less than 0.05.

CI, confidence interval; HCC, hepatocellular carcinoma; OR, odds ratio.

allele: OR = 1.329, 95% CI = 1.061–1.664, *p* = 0.013). The risk of HBV-related HCC in men was the same. However, for female patients, such an association was not found (Table 5).

For *miR-423* rs6505162, there was no statistically significant difference in terms of genotype/allele or genetic model analysis, either in whole or in stratification studies.

miR-499 polymorphism and clinicopathologic characteristics

Clinical characteristics, such as tumor number, tumor grade, AFP, TB, ALT, AST, and HBV-DNA, can serve as a marker for the development and prognosis of HCC. As shown

TABLE 4. COMPARISON OF GENOTYPE/ALLELE FREQUENCIES OF TWO POLYMORPHISMS IN MALE SUBJECTS

Genotypes	Controls		HCC patients		HCC patients with HBV		
	n (%)	n (%)	OR (95% CI) ^a	p-Value ^a	n (%)	OR (95% CI) ^a	p-Value ^a
<i>miR-423</i> rs6505162	<i>n</i> = 718	<i>n</i> = 809			<i>n</i> = 635		
CC	457 (63.7)	524 (64.8)	1.000		406 (64.0)	1.000	
CA	230 (32.0)	262 (32.4)	1.028 (0.816–1.294)	0.817	211 (33.2)	0.971 (0.757–1.244)	0.815
AA	31 (4.2)	23 (2.8)	0.616 (0.330–1.148)	0.127	18 (2.8)	0.636 (0.323–1.252)	0.19
Dominant model (CC vs. CA + AA)			1.075 (0.860–1.344)	0.524		1.013 (0.796–1.288)	0.919
Recessive model (CC + CA vs. AA)			0.605 (0.331–1.106)	0.102		0.649 (0.337–1.250)	0.196
C	1144 (79.7)	1310 (81.0)	1.000		1023 (80.6)	1.000	
A	292 (20.3)	308 (19.0)	0.904 (0.747–1.093)	0.297	247 (19.4)	0.943 (0.772–1.152)	0.568
<i>miR-499</i> rs3746444	<i>n</i> = 703	<i>n</i> = 810			<i>n</i> = 635		
TT	556 (79.1)	588 (72.6)	1.000		464 (73.1)	1.000	
TC	127 (18.1)	206 (25.4)	1.676 (1.286–2.183)	<0.001 ^b	160 (25.2)	1.697 (1.276–2.257)	<0.001 ^b
CC	20 (2.8)	16 (2.0)	0.706 (0.345–1.446)	0.341	11 (1.7)	0.631 (0.283–1.405)	0.260
Dominant model (TT vs. TC + CC)			1.538 (1.194–1.981)	0.001 ^b		1.541 (1.172–2.025)	0.002 ^b
Recessive model (TT + TC vs. CC)			0.629 (0.308–1.284)	0.203		0.562 (0.254–1.247)	0.157
T	1239 (88.1)	1382 (85.3)	1.000		1088 (85.7)	1.000	
C	167 (11.9)	238 (14.7)	1.329 (1.061–1.664)	0.013 ^b	182 (14.3)	1.307 (1.025–1.668)	0.031 ^b

^aOR and *p*-value were obtained after adjusting for age, gender, smoking status, and wine status.^b*p*-Value was less than 0.05.

TABLE 5. COMPARISON OF GENOTYPE/ALLELE FREQUENCIES OF TWO POLYMORPHISMS IN FEMALE SUBJECTS

Genotypes	Controls		HCC patients		HCC patients with HBV		
	n (%)	n (%)	OR (95% CI) ^a	p-Value ^a	n (%)	OR (95% CI) ^a	p-Value ^a
<i>miR-423</i> rs6505162	<i>n</i> = 273	<i>n</i> = 177			<i>n</i> = 128		
CC	195 (71.4)	119 (67.2)	1.000		85 (66.4)	1.000	
CA	67 (24.5)	51 (28.8)	0.882 (0.565–1.378)	0.582	37 (28.9)	0.897 (0.537–1.500)	0.679
AA	11 (4.1)	7 (4.0)	0.816 (0.288–2.315)	0.703	6 (4.7)	1.017 (0.323–3.198)	0.977
Dominant model (CC vs. CA + AA)			0.906 (0.592–1.386)	0.65		0.895 (0.550–1.456)	0.655
Recessive model (CC + CA vs. AA)			0.893 (0.331–2.410)	0.823		1.099 (0.371–3.257)	0.865
C	457 (83.7)	289 (81.6)	1.000		207 (80.9)	1.000	
A	89 (16.3)	65 (18.4)	1.067 (0.741–1.537)	0.728	49 (19.1)	1.113 (0.734–1.687)	0.614
<i>miR-499</i> rs3746444	<i>n</i> = 266	<i>n</i> = 174			<i>n</i> = 125		
TT	209 (78.6)	136 (78.2)	1.000		94 (75.2)	1.000	
TC	52 (19.6)	35 (20.1)	1.167 (0.704–1.936)	0.549	29 (23.2)	1.031 (0.582–1.824)	0.917
CC	5 (1.8)	3 (1.7)	1.281 (0.274–5.985)	0.753	2 (1.6)	1.147 (0.191–6.894)	0.881
Dominant model (TT vs. TC + CC)			1.143 (0.702–1.861)	0.592		1.020 (0.587–1.771)	0.945
Recessive model (TT + TC vs. CC)			1.129 (0.256–4.975)	0.873		0.893 (0.158–5.061)	0.898
T	470 (88.3)	307 (88.2)	1.000		217 (86.8)	1.000	
C	62 (11.7)	41 (11.8)	0.913 (0.589–1.416)	0.685	33 (13.2)	1.004 (0.611–1.649)	0.988

^aOR and *p*-value were obtained after adjusting for age, gender, smoking status, and wine status.

in Table 6, no significant association was observed between *miR-499* rs3746444/*miR-423* rs6505162 and clinicopathologic characteristics, including tumor number, tumor grade, AFP, ALT, AST, and HBV-DNA, except the marked relationship between *miR-499* rs3746444 and tumor size/TB.

Tumor size was one critical factor influencing HCC prognosis, and patients with tumors less than 5 cm in diameter had higher survival rates than those with tumors more than 5 cm in diameter. In our study, a significant association was observed between *miR-499* rs3746444 and tumor size (*p* = 0.001). Subjects with TC + CC genotypes had a larger tumor size, suggesting that they were more likely to get advanced HCC with a poor prognosis. Serum TB level is an important director of liver function, whose abnormality reflects the severity of diseases. We found that subjects with rs3746444 TT genotypes were less vulnerable to TB abnormality (Table 5). All these findings implied that rs3746444 could have an influence on the development and prognosis of HCC, and TT genotype/T allele was a protective factor, which is consistent with the earlier association results with HCC risk.

Discussion

HCC usually develops as a consequence of underlying liver disease and the cancer risk increases sharply in response to liver injury at the cirrhosis stage (El-Serag and Rudolph, 2007). A wide range of factors have been reported to be associated with the risk of HCC, such as chronic HBV or hepatitis C virus (HCV), which account for 80–90% of all HCCs worldwide, and alcoholic and nonalcoholic steatohepatitis-associated liver cirrhosis. These factors vary according to the geographical region. For example, chronic HBV infection is prevalent in many Asian countries and Africa, whereas

HCV is dominant in Japan and the United States (El-Serag and Rudolph, 2007).

It has also been reported that miRNAs play key roles in tumor formation. Several miRNAs that either suppress or promote tumor formation have been identified (Croce, 2009). Aberrant expression of miRNAs in liver often results in cancer development, such as *miR-125b* and *miR-122* (Liang *et al.*, 2010; Wen and Friedman, 2012). It is reported that *miR-125b* could suppress liver cancer cell growth, migration, and invasion (Liang *et al.*, 2010). In addition, several reports have indicated that SNPs in miRNAs and miRNA-target sites may influence miRNA biology and are associated with cancer risk, treatment response, and outcome (Saunders *et al.*, 2007; Hu *et al.*, 2008). So, the study of the relationship between HCC and the miRNA SNP is very valuable. Utilizing SNPs as companion diagnostics is a prospective option.

So far, *miR-423* and *miR-499* had not been included in previous HCC GWAS studies. The *miR-423* gene is located in the first intron of the gene of nuclear speckle splicing regulatory protein (NSRP1), playing a role in pre-miRNA splicing (Kim *et al.*, 2011; Smith *et al.*, 2012). Different expression patterns of *miR-423* were found in various types of cancers, such as malignant mesothelioma (Guled *et al.*, 2009), head and neck cancer (Hui *et al.*, 2010), and breast cancer (Farazi *et al.*, 2011). Remarkably, *miR-423* was upregulated in HCC. Instead of *miR-423-5p*, *miR-423-3p* could promote cell growth and regulate G1/S transition by inhibiting the expression of tumor suppressor p21Cip1/Waf1 in HCC (Lin *et al.*, 2011), which suggested that it was an oncogenic miRNA. Furthermore, rs6505162 in *miR-423-3p* was inferred to affect the expression or the process of *miR-423* instead of its secondary structure (Smith *et al.*, 2012). So, we hypothesized that this SNP could be related to the risk of HCC by increasing the expression of *miR-423*. Conversely, our results showed no significant association between rs6506162 in

TABLE 6. CLINICOPATHOLOGIC CHARACTERISTICS AND GENOTYPE/ALLELE FREQUENCIES OF *miR-499* rs3746444 AND *miR-423* rs6505162 POLYMORPHISM IN HEPATOCELLULAR CARCINOMA PATIENTS

<i>Indexes</i>	<i>Genotype</i>		χ^2	<i>p-Value</i>	<i>Allele</i>		χ^2	<i>p-Value</i>
Tumor size								
<i>miR-423</i> rs6505162								
< 5 cm	CC 135 (62.5)	CA 73 (33.8)	AA 8 (3.7)	0.575	C 343 (79.4)	T 89 (20.6)	1.073	0.300
≥ 5 cm	TT 214 (66.7)	TC 98 (30.5)	CC 9 (2.8)		T 526 (81.9)	C 116 (18.1)		
<i>miR-499</i> rs3746444								
< 5 cm	TT 170 (78.4)	TC 40 (18.4)	CC 7 (3.2)	0.001 ^a	T 380 (87.6)	C 54 (12.4)	3.060	0.080
≥ 5 cm	TT 218 (68.4)	TC 98 (30.7)	CC 3 (0.9)		T 534 (83.7)	C 104 (16.3)		
Tumor focus number								
<i>miR-423</i> rs6505162								
Single	CC 304 (64.3)	CA 153 (32.3)	AA 16 (3.4)	0.547 ^b	C 761 (80.4)	T 185 (19.6)	1.446	0.229
Multiple	TT 45 (71.4)	TC 17 (27.0)	CC 1 (1.6)		T 107 (84.9)	C 19 (15.1)		
<i>miR-499</i> rs3746444								
Single	TT 344 (72.9)	TC 120 (25.4)	CC 8 (1.7)	0.413 ^b	T 808 (85.6)	C 136 (14.4)	0.824	0.364
Multiple	TT 43 (68.3)	TC 18 (28.6)	CC 2 (3.1)		T 104 (82.5)	C 22 (17.5)		
Tumor grade								
<i>miR-423</i> rs6505162								
I-II	CC 60 (70.6)	CA 23 (27.1)	AA 2 (2.4)	0.437 ^b	C 143 (34.6)	T 27 (65.4)	1.974	0.160
III-IV	TT 188 (62.9)	TC 98 (32.8)	CC 13 (4.3)		T 474 (34.1)	C 124 (65.9)		
<i>miR-499</i> rs3746444								
I-II	TT 55 (64.7)	TC 27 (31.8)	CC 3 (3.5)	0.715 ^b	T 137 (80.6)	C 33 (19.4)	0.510	0.475
III-IV	TT 208 (68.9)	TC 85 (28.1)	CC 9 (3.0)		T 501 (82.9)	C 103 (17.1)		
AFP								
<i>miR-423</i> rs6505162								
< 20 µg/L	CC 243 (67.9)	CA 105 (29.3)	AA 10 (2.8)	0.410	C 591 (82.5)	T 125 (17.5)	1.544	0.214
≥ 20 µg/L	TT 385 (63.6)	TC 201 (33.2)	CC 19 (3.1)		T 971 (80.2)	C 239 (19.8)		
<i>miR-499</i> rs3746444								
< 20 µg/L	TT 266 (74.1)	TC 86 (24.0)	CC 7 (1.9)	0.944	T 618 (86.1)	C 100 (13.9)	0.041	0.839
≥ 20 µg/L	TT 442 (73.3)	TC 150 (24.9)	CC 11 (1.8)		T 1034 (85.7)	C 172 (14.3)		
Total bilirubin ^c								
<i>miR-423</i> rs6505162								
< 20 µg/L	CC 18.09 ± 20.84	CA 21.16 ± 42.99	AA 16.48 ± 9.88	0.520				
≥ 20 µg/L	TT 18.37 ± 1.05	TC 20.89 ± 2.27	CC 20.88 ± 2.63	0.004 ^a				

(continued)

TABLE 6. (CONTINUED)

<i>Indexes</i>	<i>Genotype</i>		χ^2	<i>p-Value</i>	<i>Allele</i>	χ^2	<i>p-Value</i>
Direct bilirubin ^c							
<i>miR-423</i> rs6505162	CC	CA			AA		0.810
	8.16±13.82	10.73±33.96			7.52±6.06		
<i>miR-499</i> rs3746444	TT	TC			CC		0.022 ^a
	8.56±0.78	10.18±1.75			8.91±1.41		
Indirect bilirubin ^c							
<i>miR-423</i> rs6505162	CC	CA			AA		0.610
	9.65±5.96	10.47±9.75			9.10±4.27		
<i>miR-499</i> rs3746444	TT	TC			CC		0.002 ^a
	9.54±0.26	10.81±0.57			11.97±1.32		
ALT ^c							
<i>miR-423</i> rs6505162	CC	CA			AA		0.893
	58.52±83.25	58.05±95.09			58.50±51.11		
<i>miR-499</i> rs3746444	TT	TC			CC		0.507
	57.69±85.39	62.29±92.69			47.35±41.87		
AST ^c							
<i>miR-423</i> rs6505162	CC	CA			AA		0.938
	62.19±74.56	62.59±95.82			60.54±50.97		
<i>miR-499</i> rs3746444	TT	TC			CC		0.406
	60.14±77.35	70.05±94.74			49.78±38.12		
HBV-DNA ^c							
<i>miR-423</i> rs6505162	CC	CA			AA		0.272
	1.80E06±4.67E06	1.72E06±6.87E06			1.16E06±2.11E06		
<i>miR-499</i> rs3746444	TT	TC			CC		0.857
	1.56E06±4.19E06	2.38E06±8.07E06			7.49E05±1.11E06		

^a*p*-Value was less than 0.05.^bFisher's exact test.^cOwing to non-normal distributions, nonparametric test was applied.

miR-423 and HCC in this studied population. Our findings are the first to demonstrate that this SNP could not influence the risk of HCC in a Chinese population, despite having potential to affect its expression or maturation.

The miR-499 gene lies in the 20th intron of the beta-myosin heavy chain 7B (Myh7b) gene and, thus, may result in cancer risk by affecting Myh7b gene function (Akkiz *et al.*, 2011) or downregulating Ets1, a proto-oncogene, by increasing expression of MMP-7 in HepG2 cells (Wei *et al.*, 2012). rs3746444 is situated in the stem region opposite the mature miR-499 sequence, which results in variation from an A: U pair to a G: U mismatch in the stem structure of miR-499 precursor (Xiang *et al.*, 2012). Available research on its effect on HCC risk is inconsistent on account of small samples and different ethnic groups. Similarly, relevant meta-analysis studies were considerably controversial. Therefore, we performed the association study in a larger sample (984 cases vs. 991 controls), and found that TC+CC genotype or C allele was associated with an increased risk of HCC and HBV-related HCC. Furthermore, we found that TC+CC were related to advanced HCC, larger tumor size, and high TB, suggesting its relationship with the development and prognosis of HCC. Consequently, we assumed that the C allele might decrease the expression of mature miR-499, increase the expression of Ets1, and, thus, promote HCC development and result in poor prognosis, which required further functional confirmation.

In conclusion, we demonstrated that miR-499 T>C was associated with genetic susceptibility and prognosis of HCC in a large sample, implying that it may serve as a predicative factor for HCC, while there is no effect of miR-423 rs6505162 in the susceptibility to HCC. Compared with current diagnosis tools, critical miRNA SNPs with susceptibility to HCC are advantageous in some aspects, such as cost effectiveness and reduced pain. We hope that this significant SNP can be beneficial to the diagnosis of HCC.

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