Genetic Testing and Molecular Biomarkers

Genetic Testing and Molecular Biomarkers: http://mc.manuscriptcentral.com/genetic-testing

Identification of miR-423 and miRNA-499 polymorphisms on affecting the risk of hepatocellular carcinoma in large-scale population

Journal:	*Genetic Testing and Molecular Biomarkers*
Manuscript ID:	GTMB-2013-0510
Manuscript Type:	Original Articles
Date Submitted by the Author:	16-Dec-2013
Complete List of Authors:	Ma, Yanyun; Fudan University, Ministry of Education Key Laboratory of Contemporary Anthropology and State Key Laboratory of Genetic Engineering, School of Life Sciences Wang, Rui; Fudan University, Department of Digestive Diseases of Huashan Hospital; Fudan University, Department of Immunology of Shanghai Medical School and Institutes of Biomedical Sciences Zhang, Jun; Fudan University, Department of Digestive Diseases of Huashan Hospital Li, Wenshuai; Fudan University, Department of Digestive Diseases of Huashan Hospital Gao, Chun-fang; Second Military Medical University, Eastern Hepatobiliary Surgery Hospital Liu, Jie; Fudan University, Department of Digestive Diseases of Huashan Hospital Wang, Jiucun; Fudan University, Ministry of Education Key Laboratory of Contemporary Anthropology and State Key Laboratory of Genetic Engineering, School of Life Sciences
Keyword:	Single nucleotide polymorphisms, Molecular Diagnosis

Table 1. The selected 2 SNP sites in miRNAs

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	ACGTTGGATGTTTTCCAAAAGCTCGGTCTG ACGTTGGATGCAAGCGGGGAGAAACTCAAG	CTCAGTCTTGCTTCCTA
rs3746444	C/T	hsa-mir-499	33578251(mat)	chr20:33578179-33578300	ACGTTGGATGGGCTGTTAAGACTTGCAGTG ACGTTGGATGGGAAGCAGCACAGACTTG	CCTCTCCACGTGAAC

Table 2.General characteristics in hepatocellular carcinoma patients and controls

	Cases (n = 984)	*Controls (n = 991)*	*P-value*
	No.(%) or mean ± SD	*No.(%) or mean ± SD*	
Age (years)	54.67 ± 11.24	59.6 ± 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)			
<5cm	217 (40.5)		
≥5cm	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 ± 86.59
AST (U/L, in 988 subjects)	62.36 ± 81.47
AFP	
<20ug/L	359 (37.3)
≥20ug/L	603 (62.7)
(ug/L, in 396 subjects)	124.01 ± 287.04 (0.6-1210)
HBV-DNA (IU/mL, in 444 subjects)	1.762E06 ± 5.452E06
	(1000-6.9E07)

Table 3. Association between genotypes/alleles of two polymorphisms and the risk of HCC/HBV-related HCC

Genotypes	*Controls*	*HCC patients*			*HCC patients with HBV*		
	no.(%)	*no.(%)*	*OR(95% CI)*[a]	*P-value*[a]	*no.(%)*	*OR(95% CI)*[a]	*P-value*[a]
miR-499 rs3746444	n = 969	n = 984			n = 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*	189 (24.9)	1.547 (1.203-1.990)	0.001*
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*		1.437 (1.128-1.831)	0.003*
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*	215 (14.1)	1.263 (1.017-1.569)	0.035*
miR-423 rs6505162	n= 991	n = 984			n =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

[a]OR and P value were all obtained after adjusting for age, gender, smoking status and wine status.

[*] P value was less than 0.05.

Table 4.Comparison of genotype/allele frequencies of two polymorphisms in male subjects

Genotypes	*Controls*	*HCC patients*			*HCC patients with HBV*		
	No.(%)	*No.(%)*	*OR(95% CI)*[a]	*P-value*[a]	*No.(%)*	*OR(95% CI)*[a]	*P-value*[a]
miR-423 rs6505162	n =718	n = 809			n = 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 (CCvs. 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
miR-499 rs3746444	n =703	n = 810			n = 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*	160 (25.2)	1.697 (1.276-2.257)	<0.001*
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*		1.541 (1.172-2.025)	0.002*
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*	182 (14.3)	1.307 (1.025-1.668)	0.031*

[a]OR and P value were all obtained after adjusting for age, gender, smoking status and wine status.

* 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*		
	no.(%)	*no.(%)*	*OR(95% CI)*[a]	*P-value*[a]	*no.(%)*	*OR(95% CI)*[a]	*P-value*[a]
miR-423 rs6505162	n = 273	n = 177			n = 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 (CCvs. 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
miR-499 rs3746444	n = 266	n = 174			n = 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

[a]OR and P value were obtained after adjusting for age, gender, smoking status and wine status..

[*] P value was less than 0.05.

Table 6.Clinicopathologic characteristics and genotype/allele frequencies of miR-499 rs3746444 and miR-423 rs6505162 polymorphism in HCC patients

Indexes		Genotype		P-value	Allele		P-value
Tumor size							
miR-423 rs6505162	CC	CA	AA	0.575	C	T	0.300
< 5cm	135 (62.5)	73 (33.8)	8 (3.7)		343 (79.4)	89 (20.6)	
≥ 5cm	214 (66.7)	98 (30.5)	9 (2.8)		526 (81.9)	116 (18.1)	
miR-499 rs3746444	TT	TC	CC	0.001*	T	C	0.080
< 5cm	170 (78.4)	40 (18.4)	7 (3.2)		380 (87.6)	54 (12.4)	
≥ 5cm	218 (68.4)	98 (30.7)	3 (0.9)		534 (83.7)	104 (16.3)	
Tumor focus number							
miR-423 rs6505162	CC	CA	AA	0.477	C	T	0.229
Single	304(64.3)	153 (32.3)	16 (3.4)		761(80.4)	185(19.6)	
Multiple	45 (71.4)	17 (27.0)	1 (1.6)		107 (84.9)	19 (15.1)	
miR-499 rs3746444	TT	TC	CC	0.598	T	C	0.364
Single	344 (72.9)	120 (25.4)	8 (1.7)		808 (85.6)	136 (14.4)	
Multiple	43 (68.3)	18 (28.6)	2 (3.1)		104 (82.5)	22 (17.5)	
Tumor grade							
miR-423 rs6505162	CC	CA	AA	0.384	C	T	0.160
I-II	60 (70.6)	23 (27.1)	2 (2.4)		143 (34.6)	27(65.4)	
III-IV	188 (62.9)	98 (32.8)	13 (4.3)		474 (34.1)	124 (65.9)	
miR-499 rs3746444	TT	TC	CC	0.766	T	C	0.475
I-II	55 (64.7)	27 (31.8)	3 (3.5)		137 (80.6)	33 (19.4)	
III-IV	208 (68.9)	85 (28.1)	9 (3.0)		501 (82.9)	103 (17.1)	

AFP							
miR-423 rs6505162	CC	CA	AA	0.410	C	T	0.214
< 20ug/L	243 (67.9)	105 (29.3)	10 (2.8)		591 (82.5)	125 (17.5)	
≥ 20ug/L	385(63.6)	201 (33.2)	19 (3.1)		971 (80.2)	239 (19.8)	
miR-499 rs3746444	TT	TC	CC	0.944	T	C	0.839
< 20ug/L	266 (74.1)	86 (24.0)	7 (1.9)		618 (86.1)	100 (13.9)	
≥ 20ug/L	442 (73.3)	150 (24.9)	11 (1.8)		1034 (85.7)	172 (14.3)	
Total bilirubin							
miR-423 rs6505162	CC	CA	AA	0.520			
	18.09 ± 20.84	21.16 ± 42.99	16.48 ± 9.88				
miR-499 rs3746444	TT	TC	CC	0.004*			
	18.37 ± 1.05	20.89 ± 2.27	20.88 ± 2.63				
Direct bilirubin							
miR-423 rs6505162	CC	CA	AA	0.810			
	8.16 ± 13.82	10.73 ± 33.96	7.52 ± 6.06				
miR-499 rs3746444	TT	TC	CC	0.022*			
	8.56 ± 0.78	10.18 ± 1.75	8.91 ± 1.41				
Indirect bilirubin							
miR-423 rs6505162	CC	CA	AA	0.610			
	9.65 ± 5.96	10.47 ± 9.75	9.10 ± 4.27				
miR-499 rs3746444	TT	TC	CC	0.002*			
	9.54 ± 0.26	10.81 ± 0.57	11.97 ± 1.32				
ALT							
miR-423 rs6505162	CC	CA	AA	0.893			

	58.52 ± 83.25	58.05 ± 95.09	58.50 ± 51.11	
miR-499 rs3746444	TT	TC	CC	0.507
	57.69 ± 85.39	62.29 ± 92.69	47.35 ± 41.87	
AST				
miR-423 rs6505162	CC	CA	AA	0.938
	62.19 ± 74.56	62.59 ± 95.82	60.54 ± 50.97	
miR-499 rs3746444	TT	TC	CC	0.406
	60.14 ± 77.35	70.05 ± 94.74	49.78 ± 38.12	
HBV-DNA				
miR-423 rs6505162	CC	CA	AA	0.272
	1.80E06 ± 4.67E06	1.72E06 ± 6.87E06	1.16E06 ± 2.11E06	
miR-499 rs3746444	TT	TC	CC	0.857
	1.56E06 ± 4.19E06	2.38E06 ± 8.07E06	7.49E05 ± 1.11E06	

* P value was less than 0.05.

Identification of miR-423 and miRNA-499 polymorphisms on affecting the risk of hepatocellular carcinoma in large-scale population

Yanyun Ma[1], Rui Wang[2,3], Jun Zhang[2], Wenshuai Li[2], Chunfang Gao[4], Jie Liu*[2,3], Jiucun Wang*[1]

[1]Ministry of Education Key Laboratory of Contemporary Anthropology and State Key Laboratory of Genetic Engineering, School of Life Sciences, Fudan University, Shanghai, China;

[2]Department of Digestive Diseases, Huashan Hospital, Fudan University, Shanghai, China;

[3]Department of Immunology of Shanghai Medical School and Institutes of Biomedical Sciences, Fudan University, Shanghai, China;

[4]Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai, China;

Running title: MiR-423 and MiR-499 Variants and Liver Cancer Risk

Corresponding author:
Jiucun Wang Ph.D.
School of Life Sciences, Fudan University
220 Handan Rd.
Shanghai 200433
China
Tel: 86-21-55665499
Fax: 86-21-65643714
E-mail: theresajcwang@gmail.com
Jie Liu M.D., Ph.D.
Huashan Hospital, Fudan University
12 Middle Wulumuqi Rd.
Shanghai 200040
China
Tel & Fax: 86-21-52888236
E-mail: jieliu@fudan.edu.cn

Abstracts

Aims: MicroRNAs (miRNAs) regulate gene expression and act as tumor suppressors or enhancers in oncogenesis. SNPs in miRNAs could alter the process 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 size samples of different types of population.

Methods: To evaluate the association of miR-499 rs3746444 and miR-423 rs6505162 with 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 genotype compared with those with TT genotype (OR = 1.372, 95% CI = 1.099-1.713, P = 0.005), so was the risk of HBV-related HCC (OR = 1.437, 95% CI = 1.128-1.831, P = 0.003). Moreover, subjects with TC+CC genotype were more vulnerable to advanced HCC with larger tumor size and/or higher TB, which suggested that 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.

Key Words: MicroRNA; polymorphism; hepatocellular carcinoma; MassARRAY

Mary Ann Liebert, Inc., 140 Huguenot Street, New Rochelle, NY 10801

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). Because it is often diagnosed at an advanced stage, HCC generally has 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 to surgical intervention(Blum 2005). Meanwhile, frequent tumor metastasis and tumor recurrence after surgical intervention often lead to poor outcome in HCC patients(Lu and others 2009). Therefore, identification of effective diagnostic and prognostic markers of HCC is of crucial significance.

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 miRNAs expression may influence the cancer formation (Calin and Croce 2006). SNPs within miRNAs could affect its transcription, processing, or target recognition and result in malignant diseases (Buonocore and others 2010; Pastinen and others 2006). 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 etc. may be correlated to susceptibility of HCC (Fan and others 2013; Guo and others 2012; Jun Zhang 2013; Lafferty-Whyte and others 2009; Wang and others 2011; Xu and others 2011)

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 and others 2012), breast cancer (Alshatwi and others 2012; Smith RA 2012), head and neck cancer (Liu and others 2010), esophageal cancer (Ye and others 2008) and ovarian cancer (Kontorovich and others 2010). Especially, miR-423 could distinguish HBV-related HCC from healthy controls, serving as a diagnosis biomarker for HBV-related HCC (Li and others 2010). Rs6505162 is on stem-loop structure of miR-423 and have potential to change expression or process of miR-423 (Duan S 2009), however, it is unclear about the relationship between miR-423 and genetic predisposition to HCC in any kind of ethic group. Although a couple of association researches of miR-499 rs374644 with risk of HCC have been performed, conflicting results have been reported due to small sample size and incomparable ethnic groups.

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

Materials and Methods

Mary Ann Liebert, Inc., 140 Huguenot Street, New Rochelle, NY 10801

Study Population

In our study, 984 HCC cases and 991 controls were enrolled from the unrelated East Chinese population sample collected from Huashan Hospital, Eastern Hepatobiliary Surgery Hospital and Taizhou Longitudinal Study as described previously (Jun Zhang 2013). In brief, All HCC patients were confirmed by pathologic or imaging certification and the controls had no history of cancer and 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 AFP levels, HBsAg status, HBV-DNA titer, ALT, AST, total bilirubin, 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, Union City, USA). For sake of the accuracy of subsequent experiments, all DNA samples were subjected to electrophoresis and concentration determination. Sequenom MassARRAY was performed genotype in these samples with clear strips in electrophoresis and above 10 ng / μL.

Genotyping

Amplification primers and extension primer of the SNPs, which were obtained by MassARRAY Assay Design, were listed in Table 1. Genotyping of the SNPs was performed by Sequenom MassARRAY technology, which was carried out by 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 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 chi-square tests were performed to detect the association between clinicopathologic characteristics and microRNAs genotypic/allelic frequencies in HCC patients. For the other quantitative variables which 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 subjects with HBsAg positive were defined as HBV-related HCC. For HCC patients, further instructions including the distribution of clinicopathologic characteristics were also cataloged, such as AFP, ALT, AST, and HBV-DNA, etc. It was shown that, age, gender and smoking status had significant difference between the case and the control, while alcohol consumption had no discrepancy between the two groups.

MiRNA-423 and miRNA-499 polymorphisms and the risk of hepatocellular

carcinoma

As 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 analyzed subsequently by binary logistic regression.

As shown in Table 3, TC and CC genotype 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). 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 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 males (TC+CC genotype: OR = 1.538, 95% CI = 1.194-1.981, P = 0.001; C allele: OR = 1.329, 95% CI = 1.061-1.664, P = 0.013). The risk of HBV-related HCC in males was the same. However, for female patients, such association was failed to be discovered (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.

MiRNA-499 polymorphism and clinicopathologic characteristics

Clinical characteristics, like tumor number, tumor grade, AFP, total bilirubin (TB), ALT, AST and HBV-DNA could evaluate development and prognosis of HCC. As shown 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 rs374644 and tumor size/ TB.

Tumor size was one critical factor influencing HCC prognosis, and patients who had tumor less than 5cm of diameter had higher survival rate than those with tumor more than 5cm of diameter. In our study, significant association was observed between miR-499 rs3746444 and tumor size (P = 0.001). Subjects with TC+CC genotypes had larger tumor size, suggesting they were more likely to get advanced HCC and poor prognosis. Serum total bilirubin 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). These findings all implied that rs3746444 could have influence on development and prognosis of HCC, and TT genotype/ T allele was a protective factor, which is consistent with above association results with HCC risk.

Discussion:

HCC normally 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 viral hepatitis B (HBV) or C (HCV), which account for 80–90% of all HCCs worldwide, and alcoholic and non-alcoholic steatohepatitis-associated liver cirrhosis. These factors vary according to the geographical region. For example, chronic hepatitis B virus (HBV) infection is prevalent in many Asian countries and Africa, whereas hepatitis C virus (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 leads to cancer development, such as miR-125b and miR-122(Liang and others 2010; Wen and Friedman 2012). It is reported that miR-125b could suppress liver cancer cell growth, migration and invasion (Liang and others 2010). In addition, several reports have indicated that SNPs in microRNAs and microRNA-target sites may influence microRNA biology and were associated with cancer risk, treatment response and outcome (Hu and others 2008; Saunders and others 2007). So the study of the relationship between HCC and the miRNA SNP is very valuable. Utilizing SNPs as companion diagnostics is prospective.

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 and others 2011; Smith RA 2012). Different expression patterns of miR-423 were found in various types of cancers, like malignant mesothelioma (Guled and others 2009), head and neck cancer (Hui and others 2010), and breast cancer (Farazi and others 2011). Remarkably, miR-423 was up-regulated in HCC. Instead of miR-423-5p, miR-423-3p could promote cell growth and regulate G1/S transition through inhibiting the expression of tumor suppressor p21Cip1/Waf1 in HCC (Lin and others 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 RA 2012). So we hypothesized that this SNP could be related to the risk of HCC through increasing the expression of miR-423. Conversely,

our results showed no significant association between rs6506162 in 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 Chinese population, despite having potential to affect its expression or maturation.

MiR-499 gene lays in the 20th intron of the beta-myosin heavy chain 7B (Myh7b) gene and thus may result in cancer risk through affecting Myh7b gene function (Akkiz H 2011) or down-regulating Ets1, one proto-oncogene, by increasing expression of MMP-7 in HepG2 cells (Wei and others 2012). Rs3746444 is situated in the stem region opposite to the mature miR-499 sequence which leads to variation from A: U pair to G: U mismatch in the stem structure of miR-499 precursor (Xiang and others 2012). Available researches on its effect on HCC risk are 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 increased risk of HCC and HBV-related HCC. Furthermore, we found that TC+CC were related with advanced HCC, related to larger tumor size and high TB, suggesting its relationship with 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 took advantages in some aspects, such as cost-effectiveness and less pain. We hope that this significant SNP can be beneficial to diagnose of HCC.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (No. 12JC1402000 and 12410705300). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References:

Akkiz H BS, Bekar A, Akgöllü E, Üsküdar O. (2011). Genetic variation in the microRNA-499 gene and hepatocellular carcinoma risk in a Turkish population: lack of any association in a case-control study. Asian Pac J Cancer Prev. **12**,3107-12.

Alshatwi AA, Shafi G, Hasan TN, Syed NA, Al-Hazzani AA, Alsaif MA, Alsaif AA. (2012). Differential expression profile and genetic variants of microRNAs sequences in breast cancer patients. PLoS One **7**,e30049.

Bartel DP. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. Cell **116**,281-97.

Bartel DP. (2009). MicroRNAs: target recognition and regulatory functions. Cell **136**,215-33.

Blum HE. (2005). Hepatocellular carcinoma: therapy and prevention. World J Gastroenterol **11**,7391-400.

Buonocore F, Hill MJ, Campbell CD, Oladimeji PB, Jeffries AR, Troakes C, Hortobagyi T, Williams BP, Cooper JD, Bray NJ. (2010). Effects of cis-regulatory variation differ across regions of the adult human brain. Hum Mol Genet **19**,4490-6.

Calin GA, Croce CM. (2006). MicroRNA signatures in human cancers. Nat Rev Cancer **6**,857-66.

Croce CM. (2009). Causes and consequences of microRNA dysregulation in cancer. Nat Rev Genet **10**,704-14.

Duan S MS, Zhang W, Dolan ME. (2009). Comprehensive analysis of the impact of SNPs and CNVs on human microRNAs and their regulatory genes. RNA Biol. **6**,412-25.

El-Serag HB, Rudolph KL. (2007). Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology **132**,2557-76.

Fan C, Chen C, Wu D. (2013). The association between common genetic variant of microRNA-499 and cancer susceptibility: a meta-analysis. Mol Biol Rep **40**,3389-94.

Farazi TA, Horlings HM, Ten Hoeve JJ, Mihailovic A, Halfwerk H, Morozov P, Brown M, Hafner M, Reyal F, van Kouwenhove M and others. (2011). MicroRNA sequence and expression analysis in breast tumors by deep sequencing. Cancer Res **71**,4443-53.

Guled M, Lahti L, Lindholm PM, Salmenkivi K, Bagwan I, Nicholson AG, Knuutila S. (2009). CDKN2A, NF2, and JUN are dysregulated among other genes by miRNAs in malignant mesothelioma -A miRNA microarray analysis. Genes Chromosomes Cancer **48**,615-23.

Guo J, Jin M, Zhang M, Chen K. (2012). A genetic variant in miR-196a2 increased digestive system cancer risks: a meta-analysis of 15 case-control studies. PLoS One **7**,e30585.

Hu Z, Chen J, Tian T, Zhou X, Gu H, Xu L, Zeng Y, Miao R, Jin G, Ma H and others. (2008). Genetic variants of miRNA sequences and non-small cell lung cancer survival. J Clin Invest

118,2600-8.

Hui AB, Lenarduzzi M, Krushel T, Waldron L, Pintilie M, Shi W, Perez-Ordonez B, Jurisica I, O'Sullivan B, Waldron J and others. (2010). Comprehensive MicroRNA profiling for head and neck squamous cell carcinomas. Clin Cancer Res **16**,1129-39.

Jun Zhang, Rui Wang, Yanyun Ma , Linqi Chen , Bohan Jin , Jiucun Wang , Chunfang Gao , Jie Liu. (2013). Association between the single nucleotide polymorphisms in miRNA196a-2 and miRNA146a and the susceptibility to hepatocellular carcinoma in Chinese population. ASIAN PACIFIC JOURNAL OF CANCER PREVENTION **14**. In press.

Kim YD, Lee JY, Oh KM, Araki M, Araki K, Yamamura K, Jun CD. (2011). NSrp70 is a novel nuclear speckle-related protein that modulates alternative pre-mRNA splicing in vivo. Nucleic Acids Res **39**,4300-14.

Kontorovich T, Levy A, Korostishevsky M, Nir U, Friedman E. (2010). Single nucleotide polymorphisms in miRNA binding sites and miRNA genes as breast/ovarian cancer risk modifiers in Jewish high-risk women. Int J Cancer **127**,589-97.

Lafferty-Whyte K, Cairney CJ, Jamieson NB, Oien KA, Keith WN. (2009). Pathway analysis of senescence-associated miRNA targets reveals common processes to different senescence induction mechanisms. Biochim Biophys Acta **1792**,341-52.

Li LM, Hu ZB, Zhou ZX, Chen X, Liu FY, Zhang JF, Shen HB, Zhang CY, Zen K. (2010). Serum microRNA profiles serve as novel biomarkers for HBV infection and diagnosis of HBV-positive hepatocarcinoma. Cancer Res **70**,9798-807.

Liang L, Wong CM, Ying Q, Fan DN, Huang S, Ding J, Yao J, Yan M, Li J, Yao M and others. (2010). MicroRNA-125b suppressesed human liver cancer cell proliferation and metastasis by directly

targeting oncogene LIN28B2. Hepatology **52**,1731-40.

Lin J, Huang S, Wu S, Ding J, Zhao Y, Liang L, Tian Q, Zha R, Zhan R, He X. (2011). MicroRNA-423 promotes cell growth and regulates G(1)/S transition by targeting p21Cip1/Waf1 in hepatocellular carcinoma. Carcinogenesis **32**,1641-7.

Liu Z, Li G, Wei S, Niu J, El-Naggar AK, Sturgis EM, Wei Q. (2010). Genetic variants in selected pre-microRNA genes and the risk of squamous cell carcinoma of the head and neck. Cancer **116**,4753-60.

Lu X, Zhao H, Yang H, Mao Y, Sang X, Miao R, Xu Y, Du S, Xu H, Chi T and others. (2009). A prospective clinical study on early recurrence of hepatocellular carcinoma after hepatectomy. J Surg Oncol **100**,488-93.

Pastinen T, Ge B, Hudson TJ. (2006). Influence of human genome polymorphism on gene expression. Hum Mol Genet **15 Spec No 1**,R9-16.

Saunders MA, Liang H, Li WH. (2007). Human polymorphism at microRNAs and microRNA target sites. Proc Natl Acad Sci U S A **104**,3300-5.

Smith RA JD, Gabrovska PN, Weinstein SR, Haupt L, Griffiths LR. (2012). A genetic variant located in miR-423 is associated with reduced breast cancer risk. Cancer Genomics Proteomics. **9**,115-8.

Wang JX, Jiao JQ, Li Q, Long B, Wang K, Liu JP, Li YR, Li PF. (2011). miR-499 regulates mitochondrial dynamics by targeting calcineurin and dynamin-related protein-1. Nat Med **17**,71-8.

Wei W, Hu Z, Fu H, Tie Y, Zhang H, Wu Y, Zheng X. (2012). MicroRNA-1 and microRNA-499 downregulate the expression of the ets1 proto-oncogene in HepG2 cells. Oncol Rep **28**,701-6.

Wen J, Friedman JR. (2012). miR-122 regulates hepatic lipid metabolism and tumor suppression. J Clin

Invest **122**,2773-6.

Xiang Y, Fan S, Cao J, Huang S, Zhang LP. (2012). Association of the microRNA-499 variants with susceptibility to hepatocellular carcinoma in a Chinese population. Mol Biol Rep **39**,7019-23.

Xing J, Wan S, Zhou F, Qu F, Li B, Myers RE, Fu X, Palazzo JP, He X, Chen Z and others. (2012). Genetic polymorphisms in pre-microRNA genes as prognostic markers of colorectal cancer. Cancer Epidemiol Biomarkers Prev **21**,217-27.

Xu Y, Liu L, Liu J, Zhang Y, Zhu J, Chen J, Liu S, Liu Z, Shi H, Shen H and others. (2011). A potentially functional polymorphism in the promoter region of miR-34b/c is associated with an increased risk for primary hepatocellular carcinoma. Int J Cancer **128**,412-7.

Ye Y, Wang KK, Gu J, Yang H, Lin J, Ajani JA, Wu X. (2008). Genetic variations in microRNA-related genes are novel susceptibility loci for esophageal cancer risk. Cancer Prev Res (Phila) **1**,460-9.

Mary Ann Liebert, Inc., 140 Huguenot Street, New Rochelle, NY 10801

Authors:

Yanyun Ma
E-mail: mayymail@gmail.com
Telephone: 86-21-150 0073 5649
Fax: 86-21-65643714

Rui Wang
E-mail: wangruiruiyangyang@163.com
Telephone: 86-21-52888234
Fax: 86-21-52888236

Jun Zhang
E-mail: archsteed@gmail.com
Telephone: 86-21-5288 8234
Fax: 86-21-52888236

Wenshuai Li
E-mail: liwenshuai_1990@163.com
Telephone: 86-21-5288 8234
Fax: 86-21-52888236

Chun-fang Gao
E-mail: gaocf1115@163.com
Tel: 86-21-81875131
Fax: 86-21-65562400

Jie Liu
E-mail: jieliu@fudan.edu.cn
Tel & Fax: 86-21-52888236

Jiucun Wang
E-mail: theresajcwang@gmail.com
Tel: 86-21-55665499
Fax: 86-21-65643714

Reviewers:
1) Momiao Xiong, E-mail: momiao.xiong@uth.tmc.edu;
2) Qingyi Wei, E-mail: qwei@mdanderson.org;
3) Mark A. Feitelson, E-mail: mfeitelson1@yahoo.com