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Original Article

MiR-146a genetic polymorphism contributes to the susceptibility to hepatocellular carcinoma in a Chinese population

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Abstract: Hepatocellular carcinoma (HCC) is the most common and highly malignant primary tumor worldwide. In the present study, we assessed the association of miR-146a, miR-196a2 and miR-499 polymorphisms with the development of hepatocellular carcinoma. During January 2014 and October 2015, 165 new HCC patients and 284 healthy control subjects were collected from the First Affiliated Hospital of Sun Yat-sen University. Genotype analysis of miR-146a, miR-196a2 and miR-499 genetic polymorphisms was done using polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) method. Using logistic regression analysis, we observed that the CG and GG genotypes of miR-146a significantly elevated the risk of hepatocellular carcinoma in comparison to the CC genotype, and the adjusted ORs (95% CI) were 1.638 (1.042-2.581) and 3.113 (1.652-5.866), respectively. The G allele of miR-146a was associated with a higher risk of developing hepatocellular carcinoma in comparison to the C allele (OR=1.739, 95% CI=1.302-2.321). However, no significant association was observed between miR-196a2 and miR-499 genetic polymorphisms and risk of hepatocellular carcinoma. In conclusion, we observed that GG and CG genotypes and G allele of miR-146a contribute to increased risk of hepatocellular carcinoma in the Chinese population investigated.

Keywords: MiR-146a, miR-196a2, miR-499, polymorphism, hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is the most common and highly malignant primary tumor worldwide. More than 80% of cases are reported to occur in developing countries, with half of these arising in China [1]. The clinical symptoms of early stage HCC are not typical, and thus most of patients are often diagnosed at their advanced stage.

The etiology of HCC has been widely studies, but its actual mechanism is still not well understood. The development of HCC is a complex process that mainly casued by many environmental and lifestyle factors, such as such as hepatitis B/C virus infection, exposure to aflatoxins, long term alcohol drinking [2-4]. In addition to these risk factors, numerous studies have focus on the role of hereditary factors in the pathogenesis of hepatocellular carcinoma, such as single nucleotide polymorphisms (SNPs) in interluekin-18, vascular endothelial growth factor, Kazal motifs gene, signal transducers and activators of transcription 4 and C-reactive protein [5-9].

The functions of microRNAs (miRNA) contribute to the tumorigenesis, progression, outcome, and drug resistance [11, 12]. MiRNA can regulate the function and expression of target genes through pairing with the 3'-UTR of target genes, and subsequently controls the protein expression and level of target genes [10]. SNPs in miRNA are reported to alter the expression of protein and therefore change the functions of miRNA [15, 16]. In the present study, we assessed the association of miR-146a, miR-196a2 and miR-499 polymorphisms with the development of hepatocellular carcinoma.

Table 1. Primers, restriction enzymes and digestive products of miR-146a, miR-196a2 and miR-499 genes

Genes	Primers (5'-3')	Restriction enzymes	Digestive products
MiR-146a	CATGGGTTGTGTCAGTGTCAGAGCT TGCCTTCTGTCTCCAGTCTTCCAA	SacI	C allele: 122 bp and 25 bp G allele: 147 bp
MiR-196a2	CCCCTTCCCTTCTCCTCCAGATA CGAAAACCGACTGATGTAACTCCG	MspI	T allele: 149 bp C allele: 125 bp and 24 bp
MiR-499	CAAAGTCTTCACTTCCCTGCCA GATGTTTAACTCCTCTCCACGTGATC	BclI	T allele: 26 bp and 120 bp C allele: 146 bp

Material and methods

The performance of this study was approved by the Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University. All HCC patients and controls signed an informed consent form before enrollment into study. The performance of this study was in line with the Declaration of Helsinki.

Patients with HCC

During January 2014 and October 2015, one hundred and sixty five new HCC patients were collected from the the First Affiliated Hospital of Sun Yat-sen University. The diagnosis of HCC was based on the pathological results. Moreover, the clinical manifestation, computed tomography and magnetic resonance imaging are also used to diagnose HCC. Patients with a history of recurrent or metastasis cancers, acute or chronic infectious diseases, end-stage liver or renal diseases or endocrine diseases were excluded from our study.

The mean age of HCC patients was 56.350 ± 7.556 years. There were 118 (71.515%) HCC patients and 47 (28.485%) healthy controls. A total of 55 (33.333%) patients had a habit of tobacco smoking, 84 (50.909%) HCC patients had a habit of alcohol drinking, and 32 (19.394%) HCC patients had a family history of cancer. 60 (36.364%) HCC patients had I-II TNM stage, and 105 (63.636%) HCC patients presented III-IV stage. 33 (20.000%) patients had grade A of Child-Pugh classification, 67 (40.606%) cases had grade B of Child-Pugh classification, and 65 (39.394%) cases had grade A of Child-Pugh classification.

Control subjects

A total of 284 control subjects were collected from the outpatient clinics. All the control subjects were confirmed without a history of cancer, digestive system diseases and endocrine diseases.

The mean age of control subjects was 54.958 ± 8.211 years. There were 160 (56.338%) males and 124 (43.662%) females. A total of 89 (31.338%) subjects had a habit of tobacco smoking, 102 (35.915%) subjects had a habit of alcohol drinking, and 12 (4.225%) subjects had a family history of cancer.

Data collection

A structured questionnaire was used to collect the demographic and lifestyle variables of HCC patients and controls, such as age, sex, tobacco smoking, alcohol drinking and family history of cancer. The clinical data were collected from medical records, such as alanine-transaminase (ALT), aspartate aminotransferase (AST), Tumor Node Metastasis (TNM) stage and Child-Pugh classification. Cigarette smoking was defined as who smokes at least 20 cigarettes within a week and lasts for 6 months. Alcohol drinking was defined as who drinks at least 50 ml white spirit or 500 ml beer within a week and lasts for 6 months.

DNA extraction and genotyping

Each subject was asked to provide 3 ml peripheral venous blood sample for DNA extraction. Extraction of genomic DNA was carried out using TIANGEN Blood DNA Kit (TIANGEN, Beijing, China) following the manufacture protocol. Genotype analysis of miR-146a, miR-196a2 and miR-499 genetic polymorphisms was done using polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) method. The primers, restriction enzymes and digestive products were shown in **Table 1**. The forward and reverse primers, restriction enzymes and digestive products are shown in **Table 1**. PCR was performed in a 25-μL mixture containing 2.5 μL 10× buffer (5 mM $MgCl_2$), 2 μL dNTP (25 mM), 1 μL each primer (25 pmol/L), 0.25 μL TaqDNA polymerase (5 U/μL), 1 μL DNA template (20 ng/μL), and 17.25 μL ddH_2O. The PCR cycles conditions were started at 94°C for

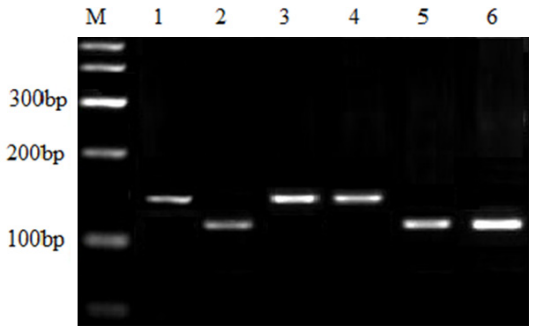

Figure 1. Genotypes for miR-146a polymorphism. Lane 1, 3 and 4 were G allele; lane 2, 5 and 6 were C allele.

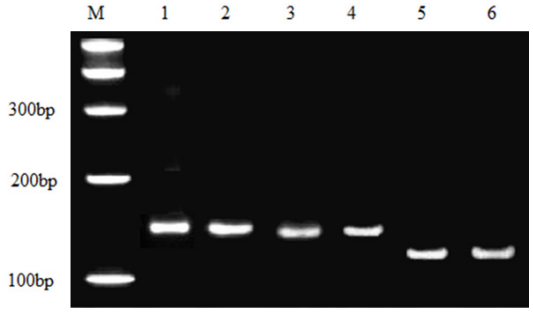

Figure 2. Genotypes for miR-196a2 polymorphism. Lane 1-4: T allele; lane 5 and 6: C allele.

5 minutes, then 30 cycles of denaturation at 94°C for 60 seconds, annealing at 62°C for 60 seconds for the miR-146a, 63°C for 60 seconds for the miR-196a2 and miR-499 extension at 72°C for 30 seconds, and a final elongation at 72°C for 10 minutes. The products were observed by the agarose gel electrophoresis and ultraviolet instrument (**Figures 1-3**).

Statistical analysis

The differences in demographic and lifestyle variables were compared by Chi-square test and Student *t*-test. The goodness-of-fit Chi-square test was taken to assess whether the genotype frequencies were in line with the Hardy-Weinberg equilibrium. Association between miR-146a, miR-196a2 and miR-499 genetic polymorphisms and hepatocellular carcinoma risk were analyzed by multiple logistic regression analysis, and the odds ratios (ORs) and corresponding 95% confidence intervals (CIs) were used to express the results. SPSS Inc. Released 2008. SPSS Statistics for Windows, Version 17.0. software (SPSS Inc. Chicago, USA) was taken to perform the statistical analysis. *P* values <0.05 was considered as statistically significance.

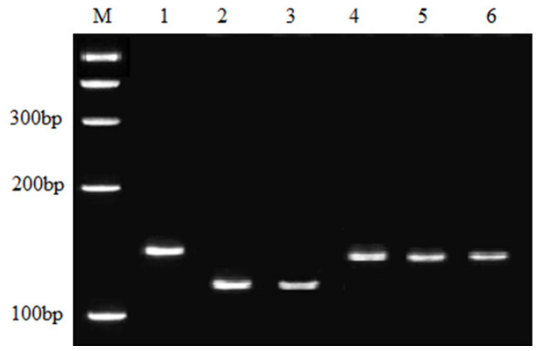

Figure 3. Genotypes for miR-499 polymorphism. Lane 1, 4-6: C allele; lane 2 and 3: T allele.

Results

Using student t test or Chi-square test, we found that HCC patients are likely to be males (χ^2=0.860, P=0.001), have a habit of alcohol consumption (χ^2=9.669, P=0.002), have a family history of cancer (χ^2=27.1669, P<0.001), and have a higher ALT (χ^2=18.242, P<0.001) and AST (χ^2=17.251, P<0.001) (**Table 2**). However, no significant differences were found between the two investigated groups in terms of age (*t*=1.549, P=0.122) and tobacco smoking (χ^2=0.191, P=0.662).

Using Chi-square test, the genotype distributions of miR-196a2 (χ^2=0.507, P=0.776) and miR-499 (χ^2=0.049, P=0.976) are comparable between the two investigated groups (**Table 3**). However, a significant difference was observed in the genotype distribution of miR-146a between the HCC patients and controls (χ^2=17.231, P<0.001). Moreover, the genotype distributions of miR-146a (χ^2=0.081, P=0.775 for patients; χ^2=0.047, P=0.828 for controls) and miR-196a2 (χ^2=0.307, P=0.580 for patients; χ^2=0.020, P=0.886 for controls) were in line with Hardy-Weinberg equilibrium in both patients and controls, while the genotype distribution of miR-499 (χ^2=4.080, P=0.043 for patients; χ^2=3.877, P=0.049 for controls) was deviation from the Hardy-Weinberg equilibrium in both patients and controls.

Using logistic regression analysis, we observed that the CG and GG genotypes of miR-146a sig-

Table 2. Demographic and clinical variables of included subjects

Variables	Patients N=165	%	Controls N=284	%	χ^2-test or t test	*P* value
Age, years	56.350 ± 7.556		54.958 ± 8.211		1.549	0.122
Sex						
Males	118	71.515	160	56.338		
Females	47	28.485	124	43.662	0.860	0.001
Tobacco smoking						
Never	110	66.667	195	68.662		
Ever	55	33.333	89	31.338	0.191	0.662
Alcohol consumption						
Never	81	49.091	182	64.085		
Ever	84	50.909	102	35.915	9.669	0.002
Family history of cancer						
No	133	80.606	272	95.775		
Yes	32	19.394	12	4.225	27.166	<0.001
ALT, mean ± SD	56.764 ± 25.212		27.811 ± 7.990		18.242	<0.001
AST, mean ± SD	57.141 ± 25.474		30.409 ± 7.547		17.251	<0.001
TNM stage						
I-II	60	36.364				
III-IV	105	63.636				
Child-Pugh classification						
A	33	20.000				
B	67	40.606				
C	65	39.394				

nificantly elevated the risk of hepatocellular carcinoma in comparison to the CC genotype, and the adjusted ORs (95% CI) were 1.638 (1.042-2.581) and 3.113 (1.652-5.866), respectively (**Table 4**). The G allele of miR-146a was associated with a higher risk of developing hepatocellular carcinoma in comparison to the C allele (OR=1.739, 95% CI=1.302-2.321). However, no significant association was observed between miR-196a2 and miR-499 genetic polymorphisms and risk of hepatocellular carcinoma.

Discussion

Single nucleotide polymorphisms (SNPs) refer to DNA sequence polymorphisms caused by a single nucleotide variation and the frequency of the genetic polymorphism is not less than 1% in a population. The mutation includes the transformation of a single base by transversion, insertion, or deletions, and the SNP is thought to result in susceptibility to human diseases and drug response [21-23]. SNPs in miRNA could regulate the expression of protein, and ultimately contribute to the growth, metastasis and drug resistance of hepatocellular carcinoma. Our study observed that the CG and GG genotypes and G allele of miR-146a played an important role in the pathogenesis of HCC.

miRNA-146a participates in regulating congenital immune response of monocytes and macrophages. TNF receptor-associated factor 6 as the target spot of miR-146a and interleukin receptor-associated kinase are associated with TNF-α pathway, and plays an important role in the pathogenesis of cancer [24]. Polymorphism of miR-146a is an G to C substitution leading to an amino acid sequence changing, and thus it could influence the expression and transcriptional regulation of miRNA.

Previous studies have reported the correlation between miR-146a genetic mutations and risk of developing many cancers, digestive system cancer, lung cancer, and cervical cancer [10-12]. Xie and Wang carried out a meta-analysis with 32 eligible studies (12,541 cases and 15,925 controls), and indicated that the miR-146a genetic mutation may contribute to the digestive system cancer risk [10]. Yin et al. performed a study with 575 lung cancer patients and 608 healthy contros, and revealed that miR-146a polymorphism was associated with the lung cancer risk in Chinese non-smoking females [11]. Ma et al. performed a study in Uygur women, and reported that allele G of miR-146a is related to the high expression of miR-146a and progression of cervical cancer [12]. However, some studies reported no correlation between miR-146a polymorphism and risk of many malignant tumors, such as papillary thyroid cancer, prostate cancer and esophageal squamous cell carcinoma [13-16].

Table 3. Genotype distributions of miR-146a, miR-196a2 and miR-499

SNPs	Patients N=165	%	Controls N=284	%	χ^2 test	*P* value	Patients		Controls	
							χ^2 test for HWE	*P* value for HWE	χ^2 test for HWE	*P* value for HWE
MiR-146a										
CC	50	30.303	129	45.423						
CG	80	48.485	126	44.366						
GG	35	21.212	29	10.211	17.231	<0.001	0.081	0.775	0.047	0.828
MiR-196a2										
TT	62	37.576	111	39.085						
TC	81	49.091	134	47.183						
CC	22	13.333	39	13.732	0.507	0.776	0.307	0.580	0.020	0.886
MiR-499										
TT	109	66.061	186	65.493						
CT	45	27.273	81	28.521						
CC	11	6.667	17	5.986	0.049	0.976	4.080	0.043	3.877	0.049

Table 4. Association between miR-146a, miR-196a2 and miR-499 genetic polymorphisms and hepatocellular carcinoma risk

	Patients N=165	%	Controls N=284	%	Adjusted OR (95% CI)[1]	*P* value
MiR-146a						
Codominant						
CC	50	30.303	129	45.423	1.0 (Ref.)	-
CG	80	48.485	126	44.366	1.638 (1.042-2.581)	0.024
GG	35	21.212	29	10.211	3.113 (1.652-5.866)	<0.001
Allele						
C	180	54.546	384	67.606	1.0 (Ref.)	-
G	150	45.455	184	32.394	1.739 (1.302-2.321)	<0.001
MiR-196a2						
Codominant						
TT	62	37.576	111	39.085	1.0 (Ref.)	-
TC	81	49.091	134	47.183	1.082 (0.700-1.676)	0.709
CC	22	13.333	39	13.732	1.010 (0.521-1.928)	0.975
Allele						
T	205	62.122	356	62.677	1.0 (Ref.)	-
C	125	37.879	212	37.324	1.024 (0.766-1.367)	0.869
MiR-499						
Codominant						
TT	109	66.061	186	65.493	1.0 (Ref.)	-
CT	45	27.273	81	28.521	0.948 (0.598-1.494)	0.810
CC	11	6.667	17	5.986	1.104 (0.450-2.604)	0.817
Allele						
T	263	159.395	453	159.507	1.0 (Ref.)	-
C	67	40.607	115	40.493	1.004 (0.704-1.423)	0.984

[1]Adjusted for age, sex, alcohol consumption, family history of cancer, ALT and AST. Ref. = Reference.

For the correlation between miR-146a polymorphism and risk of HCC in variety populations, several studies reported conflicting results [17-22]. Cong et al. performed a study in a Chinese population, and indicated that miR-146a polymorphism could affect the susceptibility to HCC, especially in HBV-infected patients [20]. Zhou et al. performed a study with 266 HCC patients and 281 healthy controls, and showed a significant association between miR-146a polymorphism and risk of HCC in China [21]. Chen et al. undertook a meta-analysis with 19 studies with 7170 cases and 9443 controls, and showed that the miR-146a and miR-196a2 polymorphisms may confer susceptibility to HCC in Asian population [23]. However, some studies reported that miR-146a did not appear to influence the susceptibility to HCC in some Asian and European populations [17-19, 22, 24]. In our study, we observed that miR-146a CG and GG genotypes and G allele was a significant correlated with the susceptibility to HCC in a Chinese population. The discrepancies of these studies may be caused by differences in ethnicities, patients and controls selection, and sample sizes.

In conclusion, we observed that GG and CG genotypes and G allele of miR-146a contribute to increased risk of hepatocellular carcinoma in the Chinese population investigated. Further studies with more samples are required to elucidate the association between miR-146a, miR-196a2 and miR-499 polymorphisms and HCC risk.

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Disclosure of conflict of interest

None.

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