Contributions of polymorphisms in miR146a, miR196a, and miR499 to the development of hepatocellular carcinoma

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ABSTRACT. Hepatocellular carcinoma is one of the most common malignant tumors worldwide; it is estimated that there were 782,000 new cases in 2012. MicroRNAs (miRNAs) play an important role in carcinogenesis by regulating oncogenes and tumor suppressors. We investigated the role of miR-146a, miR-196a2, and miR-499 polymorphisms in the risk of hepatocellular carcinoma in a Chinese population. Hepatocellular carcinoma patients (175) and healthy controls (302) were recruited between April 2013 and March 2015. Genotype analysis of miR-146a, miR-196a2, and miR-499 polymorphisms was carried out by polymerase chain reaction-restriction fragment length polymorphism. There was a significant difference between the genotype distribution of miR-196a2 in hepatocellular carcinoma patients and controls ($\chi^2 = 17.23$, $P < 0.001$). CG and GG miR-146a

genotypes significantly elevated the risk of hepatocellular carcinoma compared with the CC genotype, with adjusted ORs (95%CI) of 3.05 (1.07-8.70) and 4.96 (1.64-14.97), respectively. In the recessive model, the CG + GG genotype had a 3.75-fold risk of hepatocellular carcinoma compared with the CC genotype, with an adjusted OR (95%CI) of 3.75 (1.39-10.11). However, no significant association was observed between miR-196a2 and miR-499 variants and risk of hepatocellular carcinoma in the co-dominant, dominant, and recessive models. The miR-146a polymorphism is a G to C substitution that causes a mismatch in the stem-loop of miRNA, which influences how the expression and transcriptional regulation of miRNA affects its target genes. Our study revealed that the GG and CG genotypes of miR-146a increased the risk of hepatocellular carcinoma in the Chinese population.

Key words: miR-146a; miR-196a2; miR-499; Polymorphism; Hepatocellular carcinoma

INTRODUCTION

Hepatocellular carcinoma is one of the most common malignant tumors worldwide; it is estimated that there were 782,000 new cases in 2012 (5.6% of the total number of new hepatocellular carcinoma cases). More than 80% of cases occur in developing countries, with half arising in China (International Agency for Research on Cancer, 2012). The etiology of the disease has been widely studied, and previous research has demonstrated that many environmental and lifestyle factors, such as hepatitis B/C virus infection, aflatoxins, long-term alcohol consumption and liver cirrhosis (Nguyen et al., 2009; Liu et al., 2012; Niu et al., 2016). Previous studies have demonstrated that many genetic variations contribute to the risk of developing hepatocellular carcinoma, including C-reactive protein, Holliday junction recognition protein (HJURP), epidermal growth factor 61A/G, and Ghrelin and FasL genes (Lao et al., 2015; Shen et al., 2015b; Zhang et al., 2015a; Huang et al., 2016; Khalifa et al., 2016).

Many studies on the association between microRNAs (miRNAs) and hepatocellular carcinoma have reported that miRNAs may play an important role in cancer initiation, progression, outgrowth, and drug resistance (Anwar et al., 2013; Wojcicka et al., 2014). miRNAs belongs to a class of non-coding small RNAs that comprise 18-23 nucleotides (Reddy, 2015). Previous studies have indicated that many miRNAs play an important role in carcinogenesis by regulating the expression of oncogenes and tumor suppressors (Zhang et al., 2015c; Zhao et al., 2016). Polymorphisms in miRNAs can alter the expression of proteins, thereby changing their function (Lu et al., 2016; Song et al., 2016). Many studies have reported an association between miR-146a, miR-196a2, and miR-499 genetic polymorphisms and the development of hepatocellular carcinoma, but the results are conflicting and inconclusive (Hu et al., 2013; Shan et al., 2013; Zhou et al., 2014; Li et al., 2015a). We carried out a study to investigate the association between miR-146a, miR-196a2, and miR-499 genetic variations and the risk of developing hepatocellular carcinoma in a Chinese population.

MATERIAL AND METHODS

Subjects

We used a case-control design for this study. A total of 175 newly confirmed hepatocellular carcinoma patients and 302 healthy control subjects were recruited from the First Affiliated Hospital of Nanjing Medical University between April 2013 and March 2015. Hepatocellular carcinoma patients were diagnosed by biopsy or resected tissues were examined by a pathologist. All diagnoses were confirmed for the purposes of the study. The exclusion criteria were patients who had a history of other malignant tumors or recurrent tumors.

Control subjects were recruited from the outpatient clinics or those attending the hospital for a regular health examination. The exclusion criteria for control subjects were those with malignant tumors, end-stage liver or renal diseases, or endocrine or digestive system diseases.

Detailed environmental, lifestyle, or clinical data were selected from medical records. The environmental and lifestyle characteristics included age, gender, cigarette smoking, alcohol consumption, and history of cancer in first relatives. The included clinical factors were alanine-transaminase (ALT), aspartate aminotransferase (AST), tumor node metastasis stage, and Child-Pugh classification. Cigarette smoking was classified into those that smoked (ever) and those that did not (never), and alcohol consumption was divided into those that drank (ever) and those that did not (never).

Each subject agreed to take part in the study and signed informed consent before recruitment. We obtained permission to conduct our study from the Ethics Committee of Jiangsu Provincial Hospital.

DNA extraction and genotyping

DNA was extracted from a peripheral venous blood sample (5 mL) obtained from each subject after enrollment into the study. The blood samples were put into tubes containing 0.5 mg/mL ethylenediaminetetraacetic acid as an anticoagulant. Extraction of genomic DNA was carried out using a TIANGEN blood DNA kit (TIANGEN Biotech Co., Ltd., Beijing, China). Genotype analysis of miR-146a, miR-196a2, and miR-499 polymorphisms was carried out by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The primers and restriction enzymes are shown in Table 1 and Figure 1. The PCR cycling conditions were as follows: an initial denaturation at 95°C for 10 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 30 s; and a final extension at 72°C for 10 min. The PCR products of miR-146a, miR-196a2, and miR-499 were digested with *Sac*I, *Msp*I, and *Bcl*I restriction enzymes, respectively. The products were confirmed by electrophoresis on 1.5% agarose gel, and were observed under an ultraviolet instrument.

Statistical analysis

Comparisons of the subjects' characteristics were carried out using the chi-square (χ^2) test. Conformity of genotype frequencies to Hardy-Weinberg equilibrium was assessed by a goodness-of-fit chi-square test. The relationships between miR-146a, miR-196a2, and miR-499 polymorphisms and hepatocellular carcinoma risk were assessed using unconditional single

factor and binary logistic regression analysis, and the odds ratios (ORs) and corresponding 95% confidence intervals (95%CIs) were obtained. Spearman interaction analysis was used to investigate the relationship between the miR-146a polymorphism and demographic and lifestyle data. The SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) was used to analyze the results.

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

Genes	SNP	Primers (5'-3')	Restriction enzymes
miR-146a	rs2910164	CATGGGTTGTGTCAGTGTCAGAGCT TGCCTTCTGTCTCCAGTCTTCCAA	*Sac*I
miR-196a2	rs11614913	CCCCTTCCCTTCTCCTCCAGATA CGAAAACCGACTGATGTAACTCCG	*Msp*I
miR-499	rs3746444	CAAAGTCTTCACTTCCCTGCCA GATGTTTAACTCCTCTCCACGTGATC	*Bcl*I

SNP = single nucleotide polymorphism.

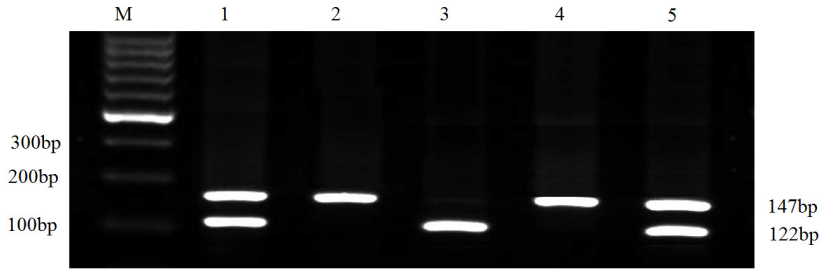

Figure 1. Agarose gel electrophoresis images for miR-146a. *Lanes 2* and *4*: G allele; *Lanes 1*, *3*, and *5*: C allele.

RESULTS

Comparisons of the subjects' characteristics are shown in Table 2. The mean ages of the hepatocellular carcinoma patients and control subjects were 56.13 ± 7.60 and 54.96 ± 8.21 years, respectively. The hepatocellular carcinoma patients and control subjects were comparable in respect of age (t = 0.86, P = 0.356) and tobacco smoking (χ^2 = 0.62, P = 0.431). However, the hepatocellular carcinoma group had more males (χ^2 = 9.16, P = 0.002), consumers of alcohol (χ^2 = 9.21, P = 0.002), individuals with a family history of cancer in first relatives (χ^2 = 26.93, P < 0.001), and had higher ALT (χ^2 = 416.86, P < 0.001) and AST (t = 399.72, P < 0.001).

We then calculated the genotype distributions of miR-146a, miR-196a2, and miR-499 between the two study groups, as shown in Table 3. There was a significant difference between the genotype distribution of miR-196a2 between hepatocellular carcinoma patients and control subjects according to the results of the chi-square test (χ^2 = 17.23, P < 0.001), whereas no significant differences were found in the genotype frequencies of miR-196a2 (χ^2 = 0.51, P = 0.776) and miR-499 (χ^2 = 0.05, P = 0.976) between the two study groups. The genotype distributions of miR-146a, miR-196a2, and miR-499 were in agreement with the HWE in patients and controls.

Table 2. Subjects' demographic, lifestyle, and clinical data.

Variables	Patients (N = 175)	%	Controls (N = 302)	%	χ^2-test or *t*-test	P value
Age, years	56.13 ± 7.60		54.96 ± 8.21		0.86	0.356
Gender						
Males	123	70.29	170	56.29		
Females	52	29.71	132	43.71	9.16	0.002
Tobacco smoking						
Never	115	65.71	209	69.21		
Ever	60	34.29	93	30.79	0.62	0.431
Alcohol consumption						
Never	87	49.71	193	63.91		
Ever	88	50.29	109	36.09	9.21	0.002
Family history of cancer						
No	142	81.14	289	95.70		
Yes	33	18.86	13	4.30	26.93	<0.001
ALT, U/L						
<40	5	2.86	294	97.35		
≥40	170	97.14	8	2.65	416.86	<0.001
AST, U/L						
<40	6	3.43	289	95.70		
≥40	169	96.57	13	4.30	399.72	<0.001
TNM stage						
I-II	62	35.43				
III-IV	113	64.57				
Child-Pugh classification						
A	34	19.43				
B	70	40.00				
C	71	40.57				

ALT = alanine-transaminase; AST = aspartate aminotransferase.

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

SNPs	Patients (N = 175)	%	Controls (N = 302)	%	χ^2 test	P value	P for HWE	
							Patients	Controls
miR-146a								
CC	52	29.71	137	45.36				
CG	86	49.14	135	44.70				
GG	37	21.14	30	9.93	17.23	<0.001	0.90	0.70
miR-196a2								
TT	65	37.14	122	40.40				
TC	85	48.57	138	45.70				
CC	25	14.29	42	13.91	0.51	0.776	0.74	0.77
miR-499								
TT	115	65.71	197	65.23				
CT	49	28.00	87	28.81				
CC	11	6.29	18	5.96	0.05	0.976	0.08	0.05

HWE = Hardy-Weinberg equilibrium.

The results of logistic regression analysis of the association between miR-146a, miR-196a2, and miR-499 polymorphisms and the risk of hepatocellular carcinoma are shown in Table 4. Using single factor logistic regression analysis, we found that only the miR-146a variant was correlated with the risk of developing hepatocellular carcinoma. We found that CG and GG genotypes of miR-146a significantly elevated the risk of hepatocellular carcinoma in comparison with the CC genotype, with adjusted ORs (95%CI) of 3.05 (1.07-8.70) and 4.96 (1.64-14.97), respectively. In the recessive model, the CG + GG genotype had a 3.75-fold risk of

hepatocellular carcinoma in comparison with the CC genotype, with an adjusted OR (95%CI) of 3.75 (1.39-10.11). However, no significant association was observed between miR-196a2 and miR-499 variants and the risk of hepatocellular carcinoma in the co-dominant, dominant, and recessive models. Using Spearman interaction analysis, no significant interaction was observed between the miR-146a polymorphism and age, gender, alcohol consumption, family history of cancer, ALT, or AST (Table 5).

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

	Patients (N = 175)	%	Controls (N = 302)	%	Crude OR (95%CI)	P value	Adjusted OR (95%CI)[1]	P value
miR-146a								
Co-dominant								
CC	52	29.72	137	45.36	1.0 (Ref.)		1.0 (Ref.)	
CG	86	49.14	135	44.71	1.96 (1.13-3.41)	0.017	3.05 (1.07-8.70)	0.037
GG	37	21.14	30	9.93	3.30 (1.85-5.89)	0.000	4.96 (1.64-14.97)	0.004
Dominant								
CC	52	29.72	137	45.36	1.0 (Ref.)		1.0 (Ref.)	
CG + GG	123	70.28	165	54.64	1.97 (1.33-2.93)	0.001	2.06 (0.96-4.42)	0.065
Recessive								
CC + CG	138	78.86	272	90.07	1.0 (Ref.)		1.0 (Ref.)	
GG	37	21.14	30	9.93	2.43 (1.44-4.11)	0.001	3.75 (1.39-10.11)	0.009
miR-196a2								
Co-dominant								
TT	65	37.14	122	40.40	1.0 (Ref.)		1.0 (Ref.)	
TC	85	48.57	138	45.70	1.20 (0.66-2.17)	0.549	0.87 (0.28-2.69)	0.810
CC	25	14.29	42	13.90	1.00 (0.56-1.78)	1.000	0.94 (0.29-2.99)	0.911
Dominant								
TT	65	37.14	122	40.4	1.0 (Ref.)		1.0 (Ref.)	
TC + CC	110	62.86	180	59.6	1.18 (0.80-1.73)	0.42	0.91 (0.44-1.88)	0.81
Recessive								
TT + TC	150	85.71	260	86.1	1.0 (Ref.)		1.0 (Ref.)	
CC	25	14.29	42	13.9	1.06 (0.61-1.81)	0.85	0.88 (0.30-2.54)	0.88
miR-499								
Co-dominant								
TT	115	65.71	197	65.23	1.0 (Ref.)		1.0 (Ref.)	
CT	49	28	87	28.81	1.01 (0.45-2.24)	0.990	0.56 (0.10-3.24)	0.561
CC	11	6.29	18	5.96	1.02 (0.44-2.37)	0.965	0.65 (0.11-4.00)	0.650
Dominant								
TT	115	65.71	197	65.23	1.0 (Ref.)		1.0 (Ref.)	
CT + CC	60	34.29	105	34.77	0.98 (0.66-1.45)	0.90	0.82 (0.38-1.76)	0.609
Recessive								
TT + CT	164	93.71	284	94.04	1.0 (Ref.)		1.0 (Ref.)	
CC	11	6.29	18	5.96	1.06 (0.48-2.32)	0.89	0.68 (0.13-3.63)	0.650

[1]Adjusted for age, gender, alcohol consumption, family history of cancer, alanine-transaminase (ALT), and aspartate aminotransferase (AST); Ref = Reference.

Table 5. Spearman interaction analysis for the relationship between the miR-146a polymorphism and demographic and lifestyle data.

Variables	Spearman correlation coefficient	P value
Age	0.059	0.114
Gender	-0.041	0.212
Alcohol consumption	0.065	0.084
Family history of cancer	0.046	0.139
ALT	0.053	0.114
AST	0.056	0.093

ALT = alanine-transaminase; AST = aspartate aminotransferase.

CONCLUSION

In this study, we evaluated the relationship between miR-146a, miR-196a2, and miR-499 genetic polymorphisms and hepatocellular carcinoma risk, and found that the CG and GG genotypes of miR-146a contributed to the pathogenesis of the disease.

The miR-146a polymorphism is a G-to-C substitution that leads to an amino acid sequence change that causes a mismatch in the stem-loop of miRNA. One previous *in vivo* study has indicated that the GG genotype of miR-146a is associated with a high expression level of mature miR-146a, and contributes to cell proliferation and colony formation in hepatocellular cancer cells (Xu et al., 2008), whereas the CC genotype elevates the expression level of mature miR-146a (Shen et al., 2008). Therefore, a polymorphism in miR-146a could influence the expression and transcriptional regulation of miRNA with regards to its target genes.

Previous studies have reported a correlation between miR-146a genetic polymorphism and the development of a variety of cancers, such as papillary thyroid cancer, esophageal squamous cell carcinoma, bladder cancer, gastric cancer, and lung cancer (Deng et al., 2015; Liu et al., 2015; Qi et al., 2015; Shen et al., 2015a; Sodhi et al., 2015; Wei et al., 2015; Zhang et al., 2015b). Zhang et al. (2015b) carried out a study in a Chinese population with 1238 papillary thyroid cancer patients and 1275 controls, and reported no significant association between the miR-146a polymorphism and the risk of papillary thyroid cancer in the Chinese population. Shen et al. (2015a) performed a case-control study in a Chinese population with 1400 esophageal squamous cell carcinoma patients and 2185 control subjects, and suggested that the miR-146a variant did not influence esophageal squamous cell carcinoma. Wei et al. (2015) carried out a meta-analysis of eight case-control studies, and reported that the miR-146a variant might marginally contribute to a reduced risk of gastric cancer, especially in Caucasians. Sodhi et al. (2015) carried out a study on 250 lung cancer cases and 255 healthy controls, and reported that the miR-146a variant was associated with an increased risk of lung cancer. Liu et al. (2015) carried out a meta-analysis on 15 case-control studies, and reported that the miR-146a polymorphism may increase the risk of colorectal cancer.

The authors of several epidemiologic studies have reported an association between the miR-146a variant and the development of hepatocellular carcinoma in a variety of populations, but their findings are conflicting and inconclusive (Xu et al., 2008; Akkız et al., 2011; Zhang et al., 2011; Hu et al., 2013; Cong et al., 2014; Zhou et al., 2014; Li et al., 2015a,b; Qi et al., 2015; Yan et al., 2015). The authors of other studies have reported similar results. Xu et al. (2008) carried out a study on 433 males and 46 females with hepatocellular carcinoma and 504 control subjects (444 males and 60 females), and reported that the GG genotype of the miR-146a variant contributed to susceptibility to hepatocellular carcinoma. Cong et al. (2014) carried out a study on 206 hepatocellular carcinoma patients (55 females and 151 males) and 217 controls (157 females and 60 males) in a Chinese population, and indicated that the GG genotype of the miR-146a polymorphism influenced the development of hepatocellular carcinoma, especially in HBV-infected patients. Zhou et al. (2014) performed a study consisting of 166 hepatocellular carcinoma patients (55 females and 151 males) and 281 controls (157 females and 60 males), and reported that the GG genotype of miR-146a was associated with an increased risk of hepatocellular carcinoma in China. However, the authors of some studies have reported inconsistent results. Akkız et al. (2011) carried out a study consisting of 222 subjects with hepatocellular carcinoma (178 males and 44 females) and 222 cancer-free control subjects (178 males and 44 females), and reported that the miR-

146a polymorphism was not associated with the development of hepatocellular carcinoma in a Turkish population (Akkız et al., 2011). Zhang et al. (2011) carried out a study including 963 hepatocellular carcinoma patients and 829 healthy controls, and discovered that the miR-146a variant did not affect susceptibility to hepatocellular carcinoma. Li et al. (2015a) carried out a case-control study in a Chinese population consisting of 184 hepatocellular carcinoma patients (126 males and 58 females) and 184 control subjects (126 males and 58 females), and discovered that the miR-146a variant did not appear to affect genetic susceptibility to hepatocellular carcinoma. Yan et al. (2015) carried out a study consisting of 274 hepatocellular carcinoma patients (61 females and 213 males) and 328 controls (120 females and 208 males), and reported no association between the miR-146a genetic variant and hepatocellular carcinoma risk in a Chinese population. In our study, we found that the miR-146a genetic polymorphism contributes to hepatocellular carcinoma risk, which is consistent with the results of previous studies. The discrepancies between these studies may have been caused by differences in ethnicity, patient and control selection, and sample size.

Two limitations of this study should also be considered. First, we examined the associations between miR-146a, miR-196a2, and miR-499 polymorphisms and hepatocellular carcinoma risk, but did not investigate the expression levels of the miRNAs or their ability to target their associated mRNAs and influence cancer risk. Second, the sample size of our study was relatively small, which may have reduced its statistical power to reveal differences between the two investigated groups. Further studies with large-scale sample sizes are needed to confirm our findings.

In conclusion, we observed that the GG and CG genotypes of miR-146a contributed to an increased risk of hepatocellular carcinoma in the Chinese population under investigation.

Conflicts of interest

The authors declare no conflict of interest.

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