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Original Article

Predictive role of miR-146a rs2910164 (C>G), miR-149 rs2292832 (T>C), miR-196a2 rs11614913 (T>C) and miR-499 rs3746444 (T>C) in the development of hepatocellular carcinoma

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Abstract: We conducted a case-control study to evaluate the association of miR-146a rs2910164 (C>G), miR-149 rs2292832 (T>C), miR-196a2 rs11614913 (T>C) and miR-499 rs3746444 (T>C) polymorphisms with the risk of hepatocellular carcinoma. A total of 274 patients with HCC were collected between January 2013 and December 2014. The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was taken to determine the polymorphism of miR-146a C>G, miR-149 T>C, miR-196a2 T>C and miR-499 T>C. By comparing with control groups, patients with HCC were more likely to be males (OR=2.01, 95% CI=1.38-2.95), have older age (OR=1.52, 95% CI=1.09-2.13), have a history of alcohol drinking (OR=2.09, 95% CI=1.49-2.93), and be infected with HBV (OR=32.98, 95% CI=19.70-55.46) and HCV (OR=56.26, 95% CI=23.28-152.98) infection. By conditional regression analysis, individuals carrying the TC and CC genotypes of miR-196a2 T>C were found to be associated with an elevated risk of HCC compared to the TT genotype, and the adjusted odds ratio were 1.50 (1.03-2.17) and 2.86 (1.60-5.16), respectively. Moreover, the TC+CC genotype was correlated with an increased risk of HCC (OR=1.69, 95% CI=1.19-2.41) compared to the wide-type genotype. In conclusion, our results suggested that miR-196a2 T>C polymorphism is associated with HCC risk in Chinese population.

Keywords: MiR-146a C>G, miR-149 T>C, miR-196a2 T>C, miR-499 T>C, polymorphism, hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC), as a malignancy, is the seventh cause of cancer related mortality in the worldwide [1]. HCC is largely a problem of the less developed regions where 83% (50% in China alone) of the estimated 782,000 new cancer cases worldwide occurred in 2012 [1]. It is estimated that there were more than 700,000 new cases are diagnosed worldwide and unfortunately more than 600,000 cases dead due to this cancer every year [2]. The prognosis of HCC is poor, and the five-year survival rate of HCC is merely 7% [3]. It is well known that the progression of HCC is a multistage process involving the deregulation of genes that are crucial to cellular processes, such as cell cycle control, cell growth, apoptosis and cell migration. HCC is highly lethal because of its aggressive metastasis and an advanced stage at the time of diagnosis. Since the diagnosis at early stage of HCC plays an important role in the development of curative therapies, identification molecular markers contribute to the high sensitivity and specificity for the development of patients with HCC.

MicroRNAs (miRNAs) are a broad class of small non-coding RNAs, and they are usually 21-25 nucleotides in length. MiRNAs could prevent translation of their target mRNAs which are critical regulators of the transcriptome [4]. MiRNAs

Table 1. Primer sequences for miR-146a C>G, miR-149 T>C, miR-196a2 T>C and miR-499 T>C

Polymorphism	Primer sequences	Length, bp	Restriction enzyme	Restriction pattern, bp
miR-146a rs2910164 (C>G)	5'-CATGGGTTGTGTCAGTGTCAGAGCT-3'	147	*Sac*I	C allele: 122 and 26
	5'-TGCCTTCTGTCTCCAGTCTTCCAA-3'			G allele: 147
miR-149 rs2292832 (T>C)	5'-TGTCTTCACTCCCGTGCTTGTCC-3'	254	*Pvu*II	C allele: 254
	5'-TGAGGCCCGAAACACCCGTA-3'			T allele: 196 and 60
miR-196a2 rs11614913 (T>C)	5'-CCCCTTCCCTTCTCCTCCAGATA-3'	149	*Msp*I	T allele: 149
	5'-CGAAAACCGACTGATGTAACTCCG-3'			C allele: 125 and 24
miR-499 rs3746444 (T>C)	5'-CAAAGTCTTCACTTCCCTGCCA-3'	146	*Bcl*I	T allele: 26 and 120
	5'-GATGTTTAACTCCTCTCCACGTGATC-3'			C allele: 146

can modulate critical cellular functions, including cell proliferation, differentiation and apoptosis, deregulation of which plays important roles in tumorigenesis and progression of cancers [5, 6]. Some miRNAs act as either oncogenes or cancer suppressor genes [7]. Single-nucleotide polymorphisms (SNPs) are the most common sequence variation in human genome. SNPs in miRNA genes may affect the property of the respective miRNAs in three ways: transcription of the primary transcript, pri-miRNA and pre-miRNA processing, and influencing miRNA-mRNA interactions [8]. Thus, polymorphisms in miRNAs might affect individual's cancer susceptibility. Four common functional variants in miR-146a rs2910164 (C>G), miR-149 rs2292832 (T>C), miR-196a2 rs11614913 (T>C) and miR-499 rs3746444 (T>C) were identified and implicated in the development of multiple-type cancers, including hepatocellular carcinoma. Several studies have evaluated the effect of these polymorphisms on the development of HCC [9-12]. In this study, we conducted a case-control study to evaluate the association of miR-146a rs2910164 (C>G), miR-149 rs2292832 (T>C), miR-196a2 rs11614913 (T>C) and miR-499 rs3746444 (T>C) polymorphisms with the risk of hepatocellular carcinoma.

Patients and methods

Patients

Between January 2013 and December 2014, a total of 274 patients with HCC were collected from our hospital. The diagnosis and histological grade of HCC were confirmed by two pathologists independently. The exclusion criteria were patients who had any other types of liver diseases (chronic hepatitis C, metabolic liver disease, authorimmune liver diseases), had a history of autoimmune or inflammatory diseases, or had a history of other cancers. The clinical stage was classified according to the Edmondson grading system. Liver function was assessed using the Child-Pugh scoring system. Tumor staging was determined according to the Union for International Cancer Control (UICC) criteria (7th Edition) and WHO classification (Pathology and Genetics of Tumors of the Digestive System).

A total of 328 controls were randomly selected from among individuals who underwent a general health checkup in the hospital during the same period time. All control subjects were confirmed to be HBV free, have no history of HCC or other liver diseases. Prior to the commencement of the study, a written informed consent was obtained from each participant for the acquisition and use of patient tissue samples and anonymized clinical data. The study was approved by the ethics committee of our hospital.

The demographic and clinical information of patients with coronary artery disease and control subjects were collected from a self-designed questionnaire and medical records. The demographic information included sex, age, smoking and drinking habit. The clinical characteristics included viral infection, liver cirrhosis, Child-Pugh classification and α-fetoprotein.

DNA extraction and genotyping

Five ml of fasting venous blood were drawn from all patients and control subjects after participating into this study. The blood samples were stored in tubes with ethylene diamine tet-

Table 2. Characteristics between patients with HCC and control subjects

Characteristics	Patients N=274	%	Controls N=328	%	*t* test or χ^2 test	*P* value	OR (95% CI)	*P* value
Age, years	57.35±12.65		54.24±11.45		3.16	0.001		
<55	121	44.16	179	54.57			1.0 (Ref.)	-
≥55	153	55.84	149	45.43	6.47	0.01	1.52 (1.09-2.13)	0.01
Gender								
Female	61	22.26	120	36.59			1.0 (Ref.)	-
Male	213	77.74	208	63.41	14.56	<0.001	2.01 (1.38-2.95)	<0.001
Smoking status								
No	143	52.19	188	57.32			1.0 (Ref.)	-
Yes	131	47.81	140	42.68	1.59	0.21	1.23 (0.88-1.72)	0.21
Alcohol status								
No	113	41.24	195	59.45			1.0 (Ref.)	-
Yes	161	58.76	133	40.55	19.81	0.001	2.09 (1.49-2.93)	<0.001
Viral infection								
Both negative	43	15.69	287	87.50			1.0 (Ref.)	-
HBV positive	168	61.31	34	10.37			32.98 (19.70-55.46)	<0.001
HCV positive	59	21.53	7	2.13			56.26 (23.28-152.98)	<0.001
Both positive	4	1.46	0	0	311.94	<0.001	-	-
Liver cirrhosis								
No	210	76.64						
Yes	64	23.36						
Child-Pugh classification								
A	43	15.69						
B	97	35.40						
C	135	49.27						
α-fetoprotein, ng/ml								
<100	119	43.43						
100-400	49	17.88						
>400	106	38.69						

raacetic acid (EDTA), and then the blood was centrifuged to separate the plasma content. Genomic DNA was extracted from the peripheral leukocytes using the TIANamp Blood DNA Kit (Tiangen, Beijing, China) according to the manufacture's instruction. The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was taken to determine the polymorphism of miR-146a C>G, miR-149 T>C, miR-196a2 T>C and miR-499 T>C. The primers sequences for miR-146a C>G, miR-149 T>C, miR-196a2 T>C and miR-499 T>C were shown in **Table 1**. The Cycling condition for miR-146a C>G, miR-149 T>C, miR-196a2 T>C and miR-499 T>C was performed at 94°C for 5 min for the initial denaturation, following 30 cycles of denaturation at 94°C for 60 s, annealing at 62°C for 60 s, extension at 72°C for 60 s and final extension at 72°C for 5 mins. The resulting DNA fragments were electrophoresed on 3.5% agarose gel and visualized under UV light after ethidium staining. About 5% of the samples were randomly selected to repeat genotyping, and the results of genotyping were 100% concordant.

Statistical analysis

Statistically significant differences between patients and controls for demographic characteristics were assessed by Student's *t*-test and the χ^2 test. Whether the miR-146a, miR-149, miR-196a2 and miR-499 confirmed with the Hardy-Weinberg equilibrium (HWE) was assessed by using the Chi-square test or Fisher's exact test. Logistic regression analysis was used to estimate the odds ratios (ORs) and the corresponding 95% confidence intervals (95% CIs) for the association between the four SNPs and development of HCC. The wide-type genotype was considered as the reference group for comparison. The odds ratio (OR) and 95% confi-

Table 3. Association between miR-146a C>G, miR-149 T>C, miR-196a2 T>C and miR-499 T>C and risk of HCC

SNPs	Patients N=274	%	Controls N=328	%	χ^2 test	*P* value	HWE	OR (95% CI)[1]	*P* value
miR-146a C>G									
CC	94	34.31	123	37.50				1.0 (Ref.)	-
CG	145	52.92	169	51.52				1.12 (0.78-1.62)	0.51
GG	35	12.77	36	10.98	0.89	0.64	0.05	1.27 (0.72-2.26)	0.39
CG+GG	180	65.69	205	62.50				1.15 (0.81-1.63)	0.42
miR-149 T>C									
TT	75	27.37	100	30.49				1.0 (Ref.)	-
TC	133	48.54	156	47.56				1.14 (0.77-1.69)	0.51
CC	66	24.09	72	21.95	0.83	0.66	0.45	1.22 (0.76-1.96)	0.38
TC+CC	199	72.63	228	69.51				1.16 (0.80-1.69)	0.40
miR-196a2 T>C									
TT	81	29.56	136	41.46				1.0 (Ref.)	-
TC	147	53.65	165	50.30				1.50 (1.03-2.17)	0.03
CC	46	16.79	27	8.23	15.20	<0.001	0.02	2.86 (1.60-5.16)	<0.001
TC+CC	193	70.44	192	58.54				1.69 (1.19-2.41)	0.003
miR-499 T>C									
TT	147	53.65	188	57.32				1.0 (Ref.)	-
TC	98	35.77	112	34.15				1.12 (0.78-1.61)	0.52
CC	29	10.58	28	8.54	1.13	0.57	0.06	1.32 (0.73-2.42)	0.33
TC+CC	127	46.35	140	42.68				1.16 (0.83-1.62)	0.37

[1]Adjusted for age, gender, alcohol drinking and viral infection.

dence intervals (CIs) are also evaluated. Statistical analysis was conducted using the SPSS 17.0 package (SPSS Inc., Chicago, IL, USA). P< 0.05 was considered to indicate a significant difference.

Results

The distributions of the demographic and clinical characteristics in HCC patients and control subjects are shown in **Table 2**. The mean ages of HCC patients and control subjects were 57.35±12.65 and 54.24±11.45 years, respectively. By comparing with control groups, patients with HCC were more likely to be males (OR=2.01, 95% CI=1.38-2.95), have older age (OR=1.52, 95% CI=1.09-2.13), have a history of alcohol drinking (OR=2.09, 95% CI=1.49-2.93), and be infected with HBV (OR=32.98, 95% CI=19.70-55.46) and HCV (OR=56.26, 95% CI=23.28-152.98) infection. However, no significant difference was found between patients with HCC and control subjects in terms of smoking status (OR=1.23, 95% CI=0.88-1.72). Of the 274 patients with HCC, 64 (23.36%) patients had liver cirrhosis, 43 (15.69%) had grade A of Child-Pugh classification, 97 (35.40%) had grade B of Child-Pugh classification, 135 (49.27%) had grade C of Child-Pugh classification, and 106 (38.69%) showed above 400 ng/ml/α-fetoprotein.

Genotype distributions of miR-146a C>G, miR-149 T>C, miR-196a2 T>C and miR-499 T>C gene polymorphisms in HCC patients and controls are shown in **Table 3**. We found that the genotype frequencies of miR-146a C>G (*P*=0.05), miR-149 T>C (*P*=0.45) and miR-499 T>C (*P*=0.06) were in Hardy-Weinberg equilibrium in the control group, however, the genotype frequencies of miR-196a2 T>C were not (*P*= 0.02). By conditional regression analysis, individuals carrying the TC and CC genotypes of miR-196a2 T>C were found to be associated with an elevated risk of HCC compared to the TT genotype, and the adjusted odds ratio were 1.50 (1.03-2.17) and 2.86 (1.60-5.16), respectively. Moreover, the TC+CC genotype of miR-196a2 T>C was correlated with an increased risk of HCC (OR=1.69, 95% CI=1.19-2.41) com-

Table 4. Interaction between the miR-196a2 T>C polymorphism and alcohol drinking and Viral infection in the risk of HCC

Variables	Patients			Controls			TC vs CC		TT vs CC	
	CC	TC	TT	CC	TC	TT	OR (95% CI)[1]	*P* value	OR (95% CI)[1]	*P* value
Drinking status										
No	31	53	82	57	90	138	1.08 (0.60-1.96)	0.78	1.09 (0.53-1.90)	0.74
Yes	44	80	117	43	66	90	1.18 (0.67-2.09)	0.53	1.27 (0.74-2.16)	0.35
Viral infection										
Both negative	12	20	11	88	136	63	1.08 (0.47-2.55)	0.85	1.28 (0.48-3.39)	0.58
HBV positive	46	81	41	10	16	8	1.10 (0.41-2.83)	0.83	1.11 (0.36-3.58)	0.84
HCV positive	17	32	10	2	4	1	0.94 (0.07-7.37)	0.95	1.18 (0.05-76.25)	0.90

[1]Adjusted for age and gender.

pared to the wide-type genotype. However, the miR-146a C>G, miR-149 T>C and miR-499 T>C polymorphisms showed no significant association with the development of HCC.

Further analysis was conducted to identify any association between the miR-196a2 T>C polymorphism and alcohol drinking and viral infection related to HCC (**Table 4**). However, we did not find any significant between miR-196a2 T>C polymorphism and the risk of HCC regardless of drinking status and viral infection (*P*>0.05).

Discussion

Genetic susceptibility to cancers has attracted growing attention to the study of gene polymorphisms involved in tumorigenesis. Meanwhile, studies also drew the importance of MicroRNAs in various biological processes. In the current study, we demonstrated that the TC and CC genotypes of miR-196a2 T>C polymorphism was associated with an increased risk of HCC compared to the TT genotype, which suggested that variation in miR-196a2 T>C contributes to the development of HCC.

MiR196 family comprises miR-196a-1, miR196-a2, and miR-196b. miR-196a could play important roles in tumorigenesis by targeting its putative targets, such as HOX gene, HMGA2 and annexin A1 [13]. Dysregulation of miR-196 expression has been reported in multiple cancer cell lines. Mature miR-196a is over-expressed in hepatocellular carcinoma tissues, suggesting it also play roles in the development of hepatocellular carcinoma [11]. Sequence alterations in miRNA genes, including pri-miRNAs, pre-miRNAs and mature miRNAs, could potentially affect miRNA biogenesis and activity [14].

Previous studies have indicated that the miR-196a2 T>C polymorphism affect the development of cancer susceptibility, such as acute lymphoblastic leukemia, oral cancer, lung cancer, gastric cancer, ovarian cancer and breast cancer [15-21]. Tong et al. conducted a study in a Chinese population, and found that the C/C and T/C genotypes of miR-196a2 T>C were associated with a significantly increased childhood acute lymphoblastic leukemia risk compared with the TT wide-type homozygote [15]. Fan et al. conducted a meta-analysis with seven studies, and they reported a significant association between miR-196a2 T>C and miR-146a C>G polymorphism and risk of lung cancer [17]. Xu et al. conducted a meta-analysis with four studies, and they found that miR-196a2 T>C polymorphism influences the susceptibility of lung cancer, while the miR-146a C>G and miR-149 T>C did not [18]. Ni et al. conducted a meta-analysis with 12 studies, and they demonstrated that the miR-146a C>G and miR-196a2 T>C might have effect on gastric cancer risk [19]. Qi et al. suggested that miR-146a C>G and miR-196a2 T>C may be biomarkers for predicting breast cancer risk in the Chinese population [21]. The above studies have suggested that polymorphism in miR-196a2 T>C contributes to the development of cancers.

Several previous studies have assessed the potential association of the miR-196a2 T>C polymorphism with HCC susceptibility [9-12, 22, 23]. Akkiz et al. conducted a case-control study in a Turkish population and consisted of

185 subjects with HCC and 185 cancer-free control subjects, and they reported an significant association between CC genotype of miR-196a2 T>C polymorphism and increased risk of HCC [12]. Kou et al. found that TT genotype of miR-196a2 T>C was correlated with HBV related hepatocellular carcinoma in a Chinese population [10]. Moreover, another three studies also reported that the miR-196a2 T>C polymorphism may contribute to HCC susceptibility in Chinese population [11, 22, 23]. However, some studies reported inconsistent results. Hao et al. conducted a study in a Chinese population, and they suggested that CT and TT genotypes of miR-196a2 T>C greatly significantly increased the risk of HCC [9]. One meta-analysis with five studies suggests that miR-146a C>G and miR-196a2 T>C are not associated with the risk of HCC [24]. The discrepancies of above mentioned studies may be illustrated by the following reasons: first, there were differences in genetic background and gene-environment interactions in the etiology of hepatocellular carcinogenesis; second, this happened to chance due to different populations, selection of patients and sample size.

Two limitations should be considered in our study. First, patients with hepatocellular carcinoma and control subjects were selected from only one hospital, which may cause selection bias. Moreover, the genotype distribution of miR-196a2 T>C did not confirm with the Hardy-Weinberg equilibrium in controls, which suggested that our population may not well represent the general population. Second, the sample size of our study is relatively small, which may significantly reduce the statistical power of the analysis.

In conclusion, our results suggested that miR-196a2 T>C polymorphism is associated with HCC risk in Chinese population. More well-designed studies based on larger sample sizes and more ethnic groups are still needed in the future.

Disclosure of conflict of interest

None.

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