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Original Article

Association of four common SNPs in microRNA polymorphisms with the risk of hepatocellular carcinoma

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Abstract: We conducted a case-control study to investigate genetic variants of miR-146a rs2910164, miR-196a2 rs11614913, miR-149 rs2292832 and miR-499 rs3746444 in the development of HCC in a Chinese population. This case-control study included 266 HCC patients and 266 control subjects between January 2012 and December 2013. Conditional logistical regression analysis indicated that TT genotype and T allele of miR-196a2 rs11614913 carried a 2.29-fold (95% CI = 1.30-4.05) and 1.60-fold (95% CI = 1.11-2.32) increased risk of HCC when compared with CC genotype, respectively. The subgroup analysis indicated that the effect of miR-196a2 rs11614913 polymorphism was influence by HBV infection. HBV infection subjects carrying the CT + TT genotype of miR-196a2 rs11614913 had an increased risk of HCC, and the OR (95% CI) was 2.89 (1.19-7.02). In conclusion, miR-196a2 rs11614913 polymorphism may contribute to identifying individuals, especially in HBV-infected subjects, who are at high risk for HCC.

Keywords: MicroRNA, polymorphism, hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC), as a malignancy, is the fifth cause of cancer related mortality in the worldwide, while the rank even reached up to the second in China [1]. The five-year survival rate of this cancer is merely 7% [2]. The development and 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. Multiple risk factors are highly correlated with HCC, including infection with the hepatitis B or C viruses, alcohol abuse, aflatoxin exposure, and metabolic diseases. Over the last decade, genetic alterations, which include the regulation of multiple oncogenes or tumor suppressor genes and the activation of tumorigenesis-related pathways, have also been identified as important factors in HCC.

MicroRNA (miRNA) belongs to a class of endogenously expressed, non-coding small RNA and contains about 22 nucleotides, and miRNA shows a high degree conservation of structure and function in metazoa. There are two forms of pre-miRNAs and mature miRNAs, and only the mature miRNAs mediated by the two RNase III endonucleases Dicer and Drosha play a key biological role [3]. It is reported that miRNAs play an important role in cell growth, differentiation, and apoptosis [4, 5]. Single nucleotide polymorphisms (SNPs) could affect the biogenesis and functions of the host miRNAs, and thus play an important role in the development of cancer [6-8].

Recently, several studies reported the role of four common SNPs in miRNAs and development of HCC in different populations, including miR-146a, miR-196a2, miR-149 and miR-499, but the results are inconsistent [9-11]. Identification of genetic variants could help evaluate the risk of HCC, such as identifying high-risk individuals. In this study, we conducted a case-control study to investigate genetic variants of miR-146a rs2910164, miR-196a2 rs11614913, miR-149 rs2292832 and miR-

Table 1. Genotyping assays of miR-146a rs2910164, miR-196a2 rs11614913, miR-149 rs2292832 and miR-499 rs3746444

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Restriction enzyme	PCR products
miR-146a rs2910164	CATGGGTTGTGTCAGTGTCAGAGCT	TGCCTTCTGTCTCCAGTCTTCCAA	*Sac*I	C allele: 122 bp, 25 bp; G allele: 147 bp
miR-196a2 rs11614913	CCCCTTCCCTTCTCCTCCAGATA	CGAAAACCGACTGATGTAACTCCG	*Msp*I	C allele: 125 bp, 24 bp; T allele: 149 bp
miR-149 rs2292832	TGTCTTCACTCCCGTGCTTGTCC	TGAGGCCCGAAACACCCGTA	*Pvu*II	C allele: 254 bp; T allele: 194 bp, 60 bp
miR-499 rs3746444	GAGTGACCAGGCCCCTTGTCTCTATTAG	TTGCTCTTTCACTCTCATTCTGGTGATG	*Bsr*GI	A allele: 52 bp; G allele: 27 bp, 25 bp

499 rs3746444 in the development of HCC in a Chinese population.

Subjects and methods

Study population

This case-control study included 266 HCC patients between January 2012 and December 2013. The diagnosis and histological grade of each case was confirmed by two pathologists independently. 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). 266 cancer-free controls were randomly selected from individuals visiting the same hospital for health checkups. Control subjects who had a history of tumor or any digestive system diseases were excluded from our study.

The clinical and demographic information of HCC patients and control subjects were collected from medical records, including sex, age, hypertension, diabetes mellitus, tobacco smoking, alcohol drinking, viral infection, tumor size and tumor stage. A written informed consent was gained from each included subject before entering the study group. The study was previously approved by Institute Research Ethics Committee of the First Affiliated Hospital of Chongqing Medical University.

Genotyping

Each patient and control subject provided a 5 mL peripheral venous blood sample after enrolling into this study. The collected blood samples were stored at -20°C until use. Genomic DNA was extracted from peripheral blood by the TIANamp Blood DNA kit (Tiangen Biotech Co., Ltd., Beijing, China). Genotyping of miR-146a rs2910164, miR-196a2 rs11614913, miR-149 rs2292832 and miR-499 rs3746444 was performed using polymerase chain reaction combined with a restriction fragment length polymorphism (PCR-RFLP). The primers for miR-146a rs2910164, miR-196a2 rs11614913, miR-149 rs2292832 and miR-499 rs3746444 were shown in **Table 1**. Amplification was performed under the cycling program: an initial denaturation step of 95°C for 5 minutes, then 30 cycles of annealing with denaturation at 94°C for 0.5 minute, touch-down annealing at 60°C for 0.5 min and final annealing at 72°C for 1 min, and finally an extension at 72°C for 10 min. The PCR products were verified by 2% agarose gel stained with ethidium bromide and ultraviolet light. For quality control, 10% of the samples were randomly selected to repeat genotyping, and the genotyping results showed 100% concordant.

Statistical analysis

Continuous variables are shown as mean ± standard deviation (SD), and categorical variables were shown frequencies and percentages. The differences between continuous and categorical variables were calculated by two tailed student's t-test and χ^2-test, respectively. Deviations from Hardy-Weinberg equilibrium (HWE) of genetic distributions of miR-146a rs2910164, miR-196a2 rs11614913, miR-149 rs2292832 and miR-499 rs3746444 in controls were evaluated by χ^2-test. Conditional logistic regression analysis was taken to analyze the association between miR-146a rs2910164, miR-196a2 rs11614913, miR-149 rs2292832 and miR-499 rs3746444 polymorphisms and risk of HCC, and the results were expressed by odds ratio (OR) and 95% confidence interval (CI). Homozygotes of the most

Table 2. Demographic and clinical characteristics of hepatocellular carcinoma patients and control subjects

Variables	Case	%	Control	%	χ^2 or *t* test	*P* value
Mean age, years						
< 55	119	44.74	124	46.62		
≥ 55	147	55.26	142	53.38	0.19	0.66
Gender						
Male	201	75.56	201	75.56		
Female	65	24.44	65	24.44	0.00	1.00
Hypertension						
No	230	86.47	233	87.59		
Yes	36	13.53	33	12.41	0.14	0.69
Diabetes mellitus						
No	228	85.71	236	88.72		
Yes	38	14.29	30	11.28	1.08	0.29
Tobacco smoking						
No	169	63.53	182	68.42		
Yes	97	36.47	84	31.58	1.42	0.23
Alcohol drinking						
No	140	52.63	169	63.53		
Yes	126	47.37	97	36.47	6.49	0.01
Viral infection						
Negative	140	52.63	230	86.47		
Positive-HBV	110	41.35	32	12.03		
Pogative-HCV	14	5.26	4	1.50		
Both positive HBV and HCV	2	0.75	0	0.00	72.29	< 0.001
Tumor size						
< 5 cm	166	62.41				
≥ 5 cm	100	37.59				
Tumor stage						
I-II	182	68.42				
III-IV	84	31.58				

frequent genotype of miR-146a rs2910164, miR-196a2 rs11614913, miR-149 rs2292832 and miR-499 rs3746444 were used as the reference group. All *P* values were two sided, and *P* value less than 0.05 was considered to be significant difference. All statistical analyses in this study were performed using SPSS software, version 16.0 (SPSS, Chicago, IL, USA) for Windows.

Results

Patients' demographic and clinical characteristics

A total of 266 patients diagnosed with HCC and 266 controls were enrolled in the study (**Table 2**). The HCC and control subjects were matched for age and sex, and *P* values were 0.66 and 1.00, respectively. There was no significant difference between patients and controls in terms of hypertension, diabetes mellitus, or tobacco smoking. By comparing with control subjects, HCC patients were more likely to have a habit of alcohol drinking and viral infection. Of the 266 HCC patients, 166 (62.41%) had tumors with diameters of < 5 cm, and 182 (68.42%) had tumor stage at I-II.

Genotype distributions of microRNA gene polymorphisms and its association with risk of HCC

The genotype distributions of miR-146a rs2910164, miR-196a2 rs11614913, miR-149 rs2292832 and miR-499 rs3746444 in the control group were in line with the Hardy-Weinberg Equilibrium (HWE), indicating that no population stratification or sampling bias existed (*P* > 0.05). Conditional logistical regression analysis indicated that TT genotype and T allele of miR-196a2 rs11614913 carried a 2.29-fold (95% CI = 1.30-4.05) and 1.60-fold (95% CI = 1.11-2.32) increased risk of HCC when compared with CC genotype, respectively (**Table 3**). However, miR-146a rs2910164, miR-149 rs2292832 and miR-499 rs3746444 polymorphisms were not associated with increased risk of HCC.

Interaction between microRNA gene polymorphisms and characteristics of HCC

A stratified analysis was used to assess whether the effect of miR-196a2 rs11614913 polymorphism was modified by sex, gender, alcohol consumption and viral infection (**Table 4**). The

Table 3. Association between miR-146a rs2910164, miR-196a2 rs11614913, miR-149 rs2292832 and miR-499 rs3746444 polymorphisms and risk of HCC

Gene	Case	%	Control	%	*P* value for HWE	OR (95% CI)[1]	*P* value
miR-146a rs2910164							
GG	151	56.77	166	62.41		1.0 (Ref.)	-
CG	86	32.33	81	30.45		1.17 (0.79-1.73)	0.42
CC	29	10.90	19	7.14	0.06	1.68 (0.87-3.30)	0.1
CG+CC	115	43.23	100	37.59		1.26 (0.88-1.82)	0.19
miR-196a2 rs11614913							
CC	84	31.58	113	42.48		1.0 (Ref.)	-
CT	131	49.25	123	46.24		1.43 (0.97-2.12)	0.06
TT	51	19.17	30	11.28	0.69	2.29 (1.30-4.05)	0.002
CT + TT	182	68.42	153	57.52		1.60 (1.11-2.32)	0.01
miR-149 rs2292832							
CC	91	34.21	108	40.60		1.0 (Ref.)	-
CT	130	48.87	124	46.62		1.24 (0.84-1.84)	0.25
TT	45	16.92	34	12.78	0.86	1.57 (0.90-2.75)	0.09
CT + TT	175	65.79	158	59.4		1.31 (0.91-1.90)	0.13
miR-499 rs3746444							
AA	150	56.39	166	62.41		1.0 (Ref.)	-
AG	92	34.59	83	31.20		1.23 (0.83-1.81)	0.28
GG	24	9.02	17	6.39	0.14	1.56 (0.77-3.22)	0.18
AG + GG	116	43.61	100	37.59		1.28 (0.89-1.84)	0.16

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

subgroup analysis indicated that the effect of miR-196a2 rs11614913 polymorphism was influence by HBV infection. HBV infection subjects carrying the CT + TT genotype of miR-196a2 rs11614913 had an increased risk of HCC, and the OR (95% CI) was 2.89 (1.19-7.02). However, sex, age and alcohol consumption had no effect on the association between miR-196a2 rs11614913 polymorphism and HCC risk.

Discussion

Knowing the role of genetic polymorphisms in HCC susceptibility could help reduce HCC mortality through early screening and diagnosis [9]. MiR-146a rs2910164, miR-196a2 rs11614913, miR-149 rs2292832 and miR-499 rs3746444 have gained attention as a diagnostic marker for carcinogenesis due to its roles in many biology processes, such as cellular senescence, apoptosis, and inflammation, as well as immune response [12, 13]. In this study, we found that the TT genotype and T allele of miR-196a2 rs11614913 were associated with a significantly increased risk of HCC, and that HBV infection significantly influenced the association between miR-196a2 rs11614913 polymorphism and HCC risk. Our study suggests that miR-196a2 rs11614913 polymorphism could be a diagnostic marker for HCC.

Several studies have reported a significant association between miR-196a2 rs11614913 polymorphism and several kinds of cancer risk, such as gastric cancer, lung cancer, colorectal cancer, breast cancer and renal cell cancer, although results are inconsistent [14-19]. Xu et al. conducted a study in a Chinese population and found that miR-196a2 rs11614913 polymorphism was not correlated with gastric cancer risk [14]. One meta-analysis with four studies reports that miR-196a2 rs11614913 influence the susceptibility of lung cancer [16]. Dikaiakos et al. conducted a case-control study in a Greek population, and they did not find a significant association between miR-196a2 rs11614913 polymorphism and risk of colorectal cancer [17]. Bansal et al. conducted a case-control study in an Indian population, and their multivariate analysis showed that miR-196a2

Table 4. Association between miR-196a2 rs11614913 polymorphisms and HCC risk by demographic and clinical characteristics

Variables	CC genotype		CT + TT genotype		Adjusted OR (95% CI)	*P* value
	Patients	Controls	Patients	Controls		
Mean age, years						
< 55	36	52	83	72	1.66 (0.95-2.93)	0.06
≥ 55	48	61	99	81	1.55 (0.94-2.58)	0.07
Gender						
Male	64	83	137	118	1.51 (0.98-2.31)	0.05
Female	20	30	45	35	1.93 (0.89-4.22)	0.07
Alcohol drinking						
No	45	71	95	98	1.53 (0.93-2.51)	0.07
Yes	39	42	87	55	1.70 (0.95-3.07)	0.06
Viral infection						
Negative	44	95	96	135	1.54 (0.96-2.46)	0.06
Positive-HBV	33	15	77	17	2.89 (1.19-7.02)	0.009
Pogative-HCV	6	3	8	1	0.25 (0.004-4.37)	0.26
Both positive HBV and HCV	1	0	1	0	-	-

rs11614913 polymorphism was carried a 3.2-fold increased risk of breast cancer in post-menopausal females [18]. Du et al. suggested that miR-196a2 rs11614913 polymorphism played an important role in the development of renal cell cancer [19]. The above mentioned studies suggest that miR-196a2 rs11614913 polymorphism may contribute to genetic susceptibility for cancer risk.

For the association between miR-196a2 rs11614913 polymorphism and HCC risk, several previous studies reported their association [20-22]. Kou et al. conducted a study in a Chinese population and reported that TT genotype of miR-196a2 rs11614913 polymorphism was associated with decreased risk of HCC, especially in those with HBV infection [20]. Hao et al. showed that TT genotype of miR-196a2 rs11614913 polymorphism significantly associated with decreased risk of HCC [21]. Han et al. conducted a large sample size study in a Chinese population, and they reported that miR-196a2 rs11614913 polymorphism was not significantly associated with HCC risk, but its CC genotype significantly enhanced the effect of the HBV mutation on HCC risk [22]. Two previous meta-analysis with more than ten studies reported that miR-196a2 rs11614913 polymorphism was not correlated with HCC risk [15, 23]. In our study, we found that TT genotype and T allele of miR-196a2 rs11614913 carried a 2.29- and 1.60-fold increased risk of HCC when compared with CC genotype. These inconsistent results might be due to differences between the studies in ethnicities, sources of controls, disease stages and/or sample sizes.

In a stratified analysis, our study found that the association between miR-196a2 rs11614913 polymorphism and HCC risk was modified by HBV infection, suggesting that miR-196a2 rs11614913 polymorphism may be involved in immune regulation during HBV infection. Two previous studies indicate that HBV replication modulates the expression of host cellular miRNAs, and thus miR-146a G > C polymorphisms are associated with HBV-related liver diseases [20, 22]. However, due to small sample size, we did not find significant interaction between miR-196a2 rs11614913 polymorphism and HCC in HCV-infected patients. This may be due to the small sample size and relatively low statistical power of our study.

Several limitations should be considered in our study. First, all the HCC patients and control subjects were selected from one hospital, and some level of selection bias could not be avoided. Second, other genetic polymorphisms may influence the development of HCC in addition to miR-146a rs2910164, miR-196a2 rs11614913, miR-149 rs2292832 and miR-499 rs3746444 polymorphisms. Second, the small numbers of cases and controls limited

the statistical power. Therefore, further studies with more subjects are needed to clarify the association between SNPs in miRNA and risk of HCC.

In conclusion, our study suggests that miR-196a2 rs11614913 polymorphism may affect the development of HCC, particularly in HBV infected patients. MiR-196a2 rs11614913 polymorphism may contribute to identifying individuals, especially in HBV-infected subjects, who are at high risk for HCC. Further studies with large sample size are greatly needed to confirm our results.

Disclosure of conflict of interest

None.

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