RESEARCH ARTICLE

Associations Between Three Common MicroRNA Polymorphisms and Hepatocellular Carcinoma Risk in Chinese

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Abstract

Aim: Associations between polymorphisms in miR-146aG>C, miR-196a2C>T and miR-499A>G and risk of HCC, and interaction with HBV infection in a Chinese population, were the target of the present research. Methods: The duplex polymerase-chain-reaction with confronting-two-pair primers (PCR-RFLP) was performed to determine the genotypes of the miR-146aG>C, miR-196a2C>T and miR-499A>G genotypes. Associations of polymorphisms with the risk of HCC were estimated by conditional logistic regression analysis. Results: Drinking, family history of cancer, HBsAg and HCV were risk factors for HCC. Multivariate regression analyses showed that subjects carrying the miR-196a2 CC genotype had significantly increased risk of HCC, with an adjusted OR (95% CI) of 2.18 (1.23-3.80). In addition, cases carrying the miR-196a2 C allele had a 1.64-fold increase in the risk for HCC (95%CI=1.03-2.49). The miR-196a2 CT and TT genotypes greatly significantly increased the risk of HCC in subjects with HBV infection, with adjusted ORs (95% CI) of 2.02 (1.12-3.68) and 2.69 (1.28-5.71), respectively. Conclusion: Our results demonstrate that miR-196a2 CC genotype and C allele have an important role in HCC risk in Chinese, especially in patients with HBV infection.

Keywords: MicroRNA - polymorphisms - hepatocellular carcinoma - HBV - risk

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer in men and the seventh in women worldwide, and it is the forth mortality rate with an estimated 109,242 new cases in China (IARC, 2008). Because of its high fatality rates, the incidence and mortality rate of HCC are almost equal (IARC, 2008). It is well known that chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, cirrhosis, aflatoxin B1 and excessive alcohol drinking are the main risk factors for HCC (El-Serag et al., 2007). Although many people are exposed to these risk factors of HCC, only about 10% of these exposed subjects develop HCC during their lifetime (Davila et al., 2004; Yu et al., 2004). Therefore, genetic factors may play an important role in the development of HCC, and interaction between genetic and environmental factors might be the main cause of HCC (Yu et al., 2004). MicroRNAs (imRNAs) are short, non-coding RNAs of approximately 23 nucleotides that regulate target genes (Bartel, 2004; Valencia-Sanchez et al., 2006). MicroRNAs have been demonstrated to have a role in several biochemical pathways in cell differentiation, proliferation, apoptosis and carcinogenesis (Lim et al., 2005; Wilfred et al., 2007). In the pathogenesis of HCC, miRNAs may have an important role in progression and directly contribute to cell proliferation, avoidance of apoptotic cell death and metastasis by targeting a large number of critical protein-coding genes (Huang and He, 2011).

It is reported that miR-196a2C>T and miR-499A>G polymorphisms are associated with various cancer, such as breast cancer, lung cancer, gastric cancer and esophageal cancer (Vinci et al., 2011; Wang et al., 2013; Wu et al., 2013; Wei et al., 2013; Zhang et al., 2013). miR-196C>T and miR-499A>G are associated with the susceptibility to hepatitis B-virus-related HCC (Akkız et al., 2011; Kim et al., 2012; Xiang et al., 2012), which suggested that miR-196C>T and miR-499A>G can serve as repressors for viral infection pathways and play a key regulator in host-virus interaction and regulation of viral replication. Therefore, a recent meta-analysis showed no association between miR-146aG>C and miR-499A>G and risk of HCC. In our study, we investigated the association between polymorphisms in miR-146aG>C, miR-196a2C>T and miR-499A>G and risk of HCC, and its interaction with HBV infection in a Chinese population.

Materials and Methods

Study population

A total of 285 cases with HCC diagnosed at the Shanxi Provincial People's Hospital were enrolled from January 2010 to April 2012. All the HCC patients were diagnosed based on the liver biopsy, or by the findings of at least two

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Table 1. Characteristics in Hepatocellular Carcinoma (HCC) Patients and Control Subjects

Variables	Cases N=235	%	Controls N=281	%	χ^2 or t	P value
Age (year, mean±SD)	53.7±9.6		53.2±9.9		0.58	0.28
Sex						
Male	169	71.9	176	62.6		
Female	66	28.1	105	37.4	5.09	0.024
Smoking						
No	147	62.6	185	65.8		
Yes	88	37.4	96	34.2	0.61	0.43
Drinking						
No	157	66.8	209	74.4		
Yes	78	33.2	72	25.6	3.51	0.06
Family history of cancer						
No	219	93.2	279	99.3		
Yes	16	6.8	2	0.7	11.45	0.001
Viral infection						
No	58	24.7	251	89.3		
HBsAg positive	133	56.6	28	10.0		
Anti-HCV Ab positive	36	15.3	2	0.7		
Both positive	8	3.4	0	0.0	217.87	<0.001

radiological tests of HCC including abdominal ultrasound, spiral computed tomography (CT), magnetic resonance imagine (MRI) and hepatic angiography. Residents with no evidence of hepatocellular who entered the hospital for health check-ups were enrolled into control group. Each control was pair-matched by sex and age (±5 years) to a patient with HCC. All the control subjects did not have a history of cancer, liver disease and other kidney disease, coronary artery disease, or other metabolic disorders. The protocol of our study was approved by the institutional review board at Shanxi Medical University and conducted in accordance with the Declaration of Helsinki. Written informed consent was collected from all patients.

The serum hepatitis B surface antigen (HBsAg) and anti-HCV antibody were assessed using microparticle enzyme immunoassay by commercial assay kits to determine the infection of hepatitis B or hepatitis C. The clinical characteristics of HCC subjects were collected by medical records. The demographic characteristics were collected with self-designed questionnaire, including smoking and alcohol drinking status. This study was approved by the Medical Ethnical Committee of Beijing Chaoyang Hospital of Capital Medical University, and a written informed consent form regarding the use of their blood samples for research studies was obtained from all participants.

DNA extraction and genotyping

All subjects were asked to provide 5 ml venous blood, and the blood samples were kept at -20°C until use, with EDTA 0.5 mg/ml used as anticoagulant. Genomic DNA was extracted using the TIANamp blood DNA kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. Duplex polymerase-chain-reaction with the confronting-two-pair primer (PCR-RFLP) was performed to determine the genotypes of the miR-146aG>C, miR-196a2C>T and miR-499A>G genotypes. The primers and products of miR-146aG>C, miR-196a2C>T and miR-499A>G were used as described previously (Jang et al., 2011).

Table 2. Comparison of Genotype Frequencies and Odd Ratio (OR) of Three miRNA Polymorphisms Between Cases and Controls

Genotype	Controls N=281	%	Cases N=235	%	χ^2	P value	OR(95% CI)*	P value
miR-146aG>C								
CC	97	34.52	70	29.79			1.0(Ref.)	-
CG	154	54.8	133	56.6			0.89(0.65-1.24)	0.49
GG	30	10.68	23	9.79	0.99	0.61	0.93(0.48-1.83)	0.81
C allele	348	61.92	273	58.09			1.0(Ref.)	-
G allele	214	38.08	179	38.09	0.30	0.58	1.07(0.82-1.39)	0.62
miR-196a2C>T								
CC	67	23.84	77	32.77			1.0(Ref.)	-
CT	160	56.94	126	53.62			0.69(0.45-1.05)	0.06
TT	55	19.57	32	13.62	6.60	0.037	0.51(0.28-0.90)	0.01
C allele	294	52.31	280	59.57			1.0(Ref.)	-
T allele	270	48.04	190	40.43	4.81	0.028	0.74(0.57-0.95)	0.02
miR-499A>G								
AA	204	72.6	160	68.09			1.0(Ref.)	-
AG	61	21.71	51	21.7			1.14(0.72-1.78)	0.56
GG	16	5.69	24	10.21	3.74	0.154	1.91(0.94-3.99)	0.06
A allele	469	83.45	371	78.94			1.0(Ref.)	-
G allele	93	16.55	99	21.06	3.45	0.063	1.35(0.97-1.87)	0.06

*Adjusted for sex, age and family history of cancer

The following PRC cycling conditions were used: an initial melting step of 5 min at 95 °C, followed by 35 cycles of denaturation at 94°C for 30 s, and annealing at 64°C for 30 s, with a final extension at 72°C for 10 min. Reproducibility was verified by repeat analysis of a randomly chosen subgroup of 10% of the subjects.

Statistically analysis

All statistical analyses were performed using the SPSS® statistical package, version 10.0 for windows (SPSS Inc., Chicago, IL, USA). Continuous and categorical variables were expressed as mean ± SD and n (%) of study participants, respectively. Comparisons between patients and control subjects were made using the Student's t-test and χ^2-test. The Hardy-Weinberg equilibriums between groups were compared using the χ^2-test. Conditional logistic regression was conducted to assess the effects of the miR-146aG>C, miR-196a2C>T and miR-499A>G on HCC risk, with results expressed as odds ratios (OR) and corresponding 95% confidence intervals (CI). Wald χ^2 test of the effect modification was conducted to assess the interaction between miR-146aG>C, miR-196a2C>T and miR-499A>G genetic variation and HBV and HCV infection. All P-values were two sided, and a P-value <0.05 was considered statistically significant.

Results

Of the 285 patients with HCC who were screened, 235 (82.46%) were eligible and were included in the study (169 males and 66 females). Mean age of HCC and control subjects were 53.7±9.6 and 53.2±9.9 years old, respectively (Table 1). Controls were matched to cases by age and sex. Drinking, family history of cancer, HBV and HCV infection were risk factors for HCC, with ORs (95%CI) of 1.60 (1.07-2.39), 8.51 (1.96-76.88), 18.26 (10.66-31.61) and 64.24 (15.51-558.03), respectively. There were no significant difference between cases and

Table 3. miR-196a2C>T Polymorphism and HCC Risk Stratified by HBV and HCV Infection

Genotype	Controls N=281	%	Not infected N=58	%	OR (95% CI)*	HBV N=133	%	OR (95% CI)*	HCV N=36	%	OR (95% CI)*
miR-196a2C>T											
CC	67	23.84	16	27.6	1.0(Ref.)	46	34.6	1.0(Ref.)	12	33.3	1.0(Ref.)
CT	160	56.94	32	55.2	0.84(0.41-1.75)	71	53.4	0.65(0.39-1.06)	19	52.8	0.66(0.29-1.59)
TT	55	19.57	10	17.2	0.84(0.32-2.11)	16	12	0.42(0.20-0.86)	5	13.9	0.63(0.16-2.11)
C allele	294	52.31	64	55.2	1.0(Ref.)	163	61.3	1.0(Ref.)	43	59.7222	1.0(Ref.)
T allele	270	48.04	52	44.8	0.92(0.60-1.39)	103	38.7	0.69(0.51-0.94)	29	40.2778	0.73(0.43-1.24)

*Adjusted for sex, age and family history of cancer

control subjects for smoking (*P* value=0.10).

The allele and genotype distributions of miR-146aG>C and miR-196a2C>T were in Hardy-Weinberg equilibrium in the control group (*P* value was 0.056 and 0.051), suggesting that there was no population stratification and no sampling bias, but miR-499A>G were not (*P* values were 0.34). The genotype distributions between cases and control subjects showed significant difference for miR-196a2C>T (χ^2=6.04, *p*=0.049), while distributions of miR-146aG>C and miR-499A>G were not (χ^2=2.18, p=0.34 for miR-146aG>C; χ^2=2.53, *p*=0.283 for miR-499A>G, Table 2). Multivariate regression analyses showed that subjects carrying miR-196a2 CC genotype had significantly increased the risk of HCC, with an adjusted OR (95% CI) of 2.18 (1.23-3.80). In addition, cases carrying miR-196a2 C allele had a 1.64-fold increase in the risk for HCC (95%CI=1.03-2.49). We identified a marginally significant increased risk of HCC in subjects with the miR-499GG genotype, when compared with miR-499AA carriers (OR=1.84, 95%CI=0.94-4.05).

To observe whether the effect of genetic variation was modified by HBV and HCV infection, HCC patients and controls were stratified on the basis of HBV and HCV infection status (Table 3). When compared with miR-196a2 CC genotype, subjects carrying miR-196a2 TC and CC genotype greatly significantly increased the risk of HCC for subjects with HBV infection, with adjusted ORs (95% CI) of 2.02 (1.12-3.68) and 2.69 (1.28-5.71), respectively. A significant interaction was found between miR-196a2C>T genetic variants and HBV infection (P for interaction: 0.007). However, we did not find significant association between miR-196a2C>T polymorphisms and HCC risk in patients with HCV infection or no HBV and HCV infection.

Discussion

In the present case-control study of hepatocellular cancer in a Chinese population, we found that CC genotype and C allele of the miR-196a2C>T polymorphism was associated with significantly decreased risk of HCC. Furthermore, stratified analysis showed that the differences between cases and controls were statistically significant in HBV-related HCC, but not in HCV infection and both negative HBV and HCV infection individuals, which suggested that miR-196a2C>T genetic variants have an important role in the immune regulation of HBV infection. However, we did not find signiciant association between miR-146aG>C and miR-499A>G genetic variants and risk of HCC.

Cancer is a disease induced by environment and genetic factors, and the genetic factors included abnormal changes in protein coding genes and non-coding genes such as miRNA. As far as we known, more than half of the known miRNAs are located in the cancer-associated genomic regions, and play an important role in the process of carcinogenesis and function as a tumor suppressor or oncogene (Esquela-Kerscher and Slack, 2006). Previous studies indicated miR-21, -10, -222, -224 and -18 showed increased expressions in HCC tissue compared with non-tumor tissue, while miR-26, -125, -199 an -200 presented decreased expression (Ladeiro et al., 2008; Li et al., 2008; Masaki, 2009). These miRNAs are also associated with cancer initiation and progression. There one common mechanism of altering the level of miRNAs in various cancer and induce the development of cancer, which is the single nucleotide polymorphism. SNPs in miRNA sequences may alter the expression and maturation of miRNA, and thus induce the development, progression and prognosis of caners (Wu et al., 2008).

Our study has demonstrated that the miR-196a2C>T was related with the risk of HCC. Previous studies indicated that miR-196a2C>T polymorphisms have been widely investigated in various cancers, such as non-small cell lung cancer, breast cancer, pancreatic cancer and esophageal cancer as well as gastric cancer (Zhan et al., 2012; Linhares et al., 2012; Pavlakis et al., 2013; Wang et al., 2013; Wei et al., 2013; Yuan et al., 2013). Three studies reported that miR-196a2C>T polymorphism are associated with development of HCC (Li et al., 2010; Qi et al., 2010). A study conducted in Chinese HBV-related HCC patients demonstrated that the risk of HCC was significantly higher with miR-196a2 CT genotype or T allele compared with those with the CC genotype (Qi et al., 2010). Another study with 310 HCC patients and 222 controls indicated that miR-196a2 CC genotype was associated with significantly increased mature miR-196a expression, and miR-196a2 polymorphism may contribute to cirrhosis-related HCC susceptibility in Chinese patients through influencing mature miR-196a expression (Li et al., 2010). Our study reported that miR-196a2 CC genotype and C allele significantly increased the risk of HCC when compared with CC genotype, which was in line with previous studies (Li et al., 2010; Qi et al., 2010). However, the results are inconsistent. A recent study indicated that polymorphism of miR-196a2C>T might not be a HCC susceptible factor of HCC, but could affect the effects of the HBV mutations (Han et al., 2013). The inconsistency of these studies may be explained by differences in population background, source of control subjects, sample

size, and also by chance, and therefore these results are greatly needed to confirm by further studies.

This study has several major limitations. Firstly, controls were selected from hospital visitors, which may induce selection bias in our study and reduce the validity of the results. The selection bias still exists, because these controls were not a random sample of the general population and may not fully represent the underlying population. However, the controls in our study were selected from individuals who came to our hospital for health check-up, and these controls could represent the general population. Secondly, the number of cases is relative small. The relative small sample size may decrease the statistical power to find the association between miR-146aG>C and miR-499A>G genetic variations and HCC risk. Therefore, further large sample size and multicenter studies are greatly needed to confirm the association between SNPs in miRNA and risk of HCC.

In summary, our results demonstrate that miR-196a2 CC genotype and C allele have an important role in HCC risk in a Chinese population, especially in patients with HBV infection. However, no significant association is observed between miR-146aG>C and miR-499A>G genetic polymorphisms and HCC risk. Further large sample studies are warranted to clarify the association between SNPs in miRNA sequences and the susceptibility to HCC in different ethnic populations.

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