GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

miR-499A>G rs3746444 and miR-146aG>C expression and hepatocellular carcinoma risk in the Chinese population

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Genet. Mol. Res. 12 (4): 5365-5371 (2013)
Received February 4, 2013
Accepted August 14, 2013
Published November 7, 2013
DOI http://dx.doi.org/10.4238/2013.November.7.11

ABSTRACT. We conducted a case-control study of a possible association of miR-499A>G rs3746444 and miR-146aG>C rs2910164 with risk of hepatocellular carcinoma. Samples from 172 hepatocellular carcinoma patients and 185 cancer-free controls were collected from October 2008 to December 2011. PCR-RFLP analysis was performed to determine the polymorphisms in each individual. The MAFs of miR-146aG>C and miR-499A>G in controls were similar to that known from the SNP database, and frequencies of genotypes in controls were in line with Hardy-Weinberg equilibrium. We found that miR-499 AG was significantly associated with decreased risk for hepatocellular carcinoma when compared with miR-499 AA genotype (adjusted odds ration = 0.74, 95% confidence interval = 0.24-0.96). However, subjects carrying miR-146a GG had a non-significant 0.62-fold decreased risk of hepatocellular carcinoma. We did not find a significant association of miR-146aG>C rs2910164 and miR-499A>G rs3746444 polymorphisms with hepatocellular carcinoma risk

in the Chinese population. Further investigations are warranted to clarify the relationship between miRNA polymorphisms and susceptibility to hepatocellular carcinoma risk in various ethnic populations.

Key words: miR-499A>G rs3746444; miR-146aG>C rs2910164; Hepatocellular carcinoma; Susceptibility

INTRODUCTION

Hepatocellular carcinoma (HCC) is mainly occurred in developing countries, and the fifth most common cancer and third leading cause of death from cancer worldwide (International Agency for Research on Cancer, 2008). Chronic hepatitis B virus and hepatitis C virus infections, aflatoxin B1, alcoholic and nonalcoholic steatohepatitis all contribute to the carcinogenic mechanism, but only a few infected patients develop HCC during their lifetime, which suggests other genetic and environmental factors may be involved in the development of HCC. Therefore, it is necessary to identify these genetic factors in the carcinogenic mechanism.

MicroRNAs (miRNAs) are a kind of small non-coding RNA molecules, about 22 nucleotides in length. Mature miRNAs targeting the 3'-untranslated region of mRNA suppress the translation or induce the cleavage of target RNA transcripts (Lu et al., 2009). It is reported that miRNA could be related to 200 genes, and play a key role in gene regulation and physiologic and pathologic processes, such as tumorignesis, cell proliferation, apoptosis and metabolism (He and Hannon, 2004; Krek et al., 2005; Johnnidis et al., 2008; Aumiller and Forstemann, 2008; Mocellin et al., 2009; Zhou et al., 2010). A previous experimental study indicated that MiRNA has a key influence on tumor biology and is related to the progression and prognosis of cancer, and that the high expression of miR-499A>G in serum was significantly associated with a longer survival of non-small cell lung cancer (Hu et al., 2010; Liu et al., 2011). Common single nucleotide polymorphisms in premiRNA, rs3746444 in miR-499A>G and rs2910164 in miR-146aG>C, have been studied in various cancers, such as breast cancer, gastric cancer, cervical squamous cell cancer and colorectal cancer (Catucci et al., 2010; Okubo et al., 2010; Gao et al., 2011; Zhou et al., 2011; Srivastava and Srivastava, 2012; Wang et al., 2012). However, the results of these studies were inconsistent. Therefore, the aim of our study was to confirm the association of rs3746444 in miR-499A>G and rs2910164 in miR-146aG>C with risk of HCC, and the interaction of the two miRNAs with HBV infection.

MATERIAL AND METHODS

From October 2008 to December 2011, a total of 172 HCC patients were recruited for this study. They were diagnosed by liver biopsy, or by the findings of at least two radiological tests of HCC, including abdominal ultrasound, magnetic resonance imaging (MRI), hepatic angiography and contrast-enhanced dynamic computed tomography, or by increased alpha-fetoprotein (AFP ≥200 μg/mL). Patients with secondary or recurrent tumors, and a history of other malignant tumors were excluded. A total of 185 cancer-free controls who were seen at the physical examination center in the First Affiliated Hospital of Wenzhou Medical College

were selected and matched with cases by age within 5 years and sex. Controls who had clinical liver diseases were excluded.

Serum hepatitis B surface antigen (HBsAg) and anti-HCV antibody were assayed by microparticle enzyme immunoassay using commercial kits to determine hepatitis B or hepatitis C infection. All subjects were given a questionnaire to investigate the demographic characteristics, history of cancer and alcohol and tobacco use. The clinical characteristics were collected from medical records, including tumor differentiation, tumor size, metastasis, cirrhosis, Child-Pugh class, chemotherapy and surgery.

This study was approved by the Medical Ethnical Committee of the First Affiliated Hospital of Wenzhou Medical College, and informed consent was obtained from all participants.

DNA collection and genotyping

All participants were asked to provide 5-mL blood samples, which were stored at -20°C. DNA was extracted from the buffy-coat fractions with the TIANamp blood DNA kit (Tiangen Biotech, Beijing, China). Duplex polymerase-chain reaction with the confronting-two-pair primer method (PCR-RFLP) was used to determine the genotype of polymorphisms in miR-146aG>C and miR-499A>G. PCR of the miR-146aG>C polymorphism was performed using the following primers to generate a 147-bp product: forward: 5'-CAT GGG TTG TGT CAG TGT CAG AGC T-3' and reverse: 5'-TGC CTT CTG TCT CCA GTC TTC CAA-3'. miR-499A>G polymorphism was genotyped using the following primers to amplify a 146-bp fragment: forward: 5'-CAA AGT CTT CACTTC CCT GCC A-3' and reverse: 5'-GAT GTT TAA CTC CTC TCC ACG TGATC-3'. For quality control, genotyping was performed without knowledge of the case/control status of the subjects, and a random sample of 5% of cases and controls was genotyped again by different researchers. The reproducibility was 100%.

Statistical analysis

Statistical analyses were conducted using the software Statistical Package for Social Science (SPSS) for Windows (version 13.0). Continuous variables are reported as means ± standard deviation (SD), while categorical variables were shown as frequencies and percentages. Differences between demographic and clinical characteristics were compared between cases and controls by means of the χ^2 test and Student *t*-test. The χ^2 test was performed on genotype frequencies of miR-146aG>C and miR-499A>G for Hardy-Weinberg equilibrium in controls. The associations of polymorphisms in miR-146aG>C and miR-499A>G with the risk of HCC were estimated by odds ratios (OR) and their 95% confidence interval (95%CI).

RESULTS

The demographic characteristics of the subjects included and clinical features of HCC patients are shown in Table 1. The average age was 56.5 ± 7.2 years in HCC cases, and was 57.3 ± 7.4 years in controls. There was no significant difference for gender and age ($P > 0.05$). Drinking was associated with a higher risk of HCC ($P < 0.05$), and HCC patients had a higher

proportion of positive HBsAg and positive anti-HCV than did controls ($P < 0.05$). Individuals who had a family history of cancer had a higher risk of HCC ($P < 0.05$).

The frequency distributions of different genotypes for miR-146aG>C rs2910164 and miR-499A>G rs3746444 are shown in Table 2. The minor allele frequencies in controls were similar to those in dbSNP, and the frequencies of genotypes in controls were in line with Hardy-Weinberg equilibrium, which suggested no population stratification and sample bias. We did not find a significant difference in the frequency distributions of different genotypes of miR-146aG>C and miR-499A>G between cases and controls.

Table 1. Comparison of the characteristics selected of hepatocellular carcinoma cases and controls.

Characteristics	Case (N = 172)	%	Control (N = 185)	%	P value
Age (means ± SD, years)	56.5 ± 7.2		57.3 ± 7.4		0.84
Gender					
Male	109	63.4	123	66.5	0.53
Female	63	36.6	62	33.5	
Smoking status (%)					
Smokers	47	27.2	43	23.4	0.375
Non-smokers	125	72.8	142	76.6	
Drinking status (%)					
Drinkers	56	32.5	43	23.1	<0.05
Non-drinkers	116	67.5	142	76.9	
Family history of cancer (%)					
Yes	14	8.4	3	1.4	<0.05
No	158	91.6	182	98.6	
HBsAg (%)					
+	71	41.2	16	8.4	<0.05
-	101	58.8	169	91.6	
Anti-HCV (%)					
+	9	5.2	1	0.5	<0.05
-	163	94.8	184	99.5	

HBsAg = serum hepatitis B surface antigen; HCV = hepatitis C virus.

Table 2. Distribution of miR-146aG>C and miR-499A>G genotypes in cases and controls.

Genotype	MAFs		Cases	%	Controls	%	P value	P for HWE in controls
	In dbSNP	In controls						
miR-146aG>C rs2910164								
CC	0.3814	0.3865	82	47.7	78	42.2	0.53	0.08
GC			62	36.1	71	38.3		
GG			28	16.2	36	19.5		
miR-499A>G rs3746444								
AA	0.1809	0.204	128	74.3	123	66.7	0.18	0.12
AG			37	21.5	48	25.8		
GG			7	4.2	14	7.5		

MAF = minor allele frequency; HWE = Hardy-Weinberg equilibrium.

Multivariate regression analysis was conducted to assess the role of polymorphisms in miR-146aG>C and miR-499A>G in the risk of HCC (Table 3). We found that miR-499 AG was significantly associated with decreased risk of HCC when compared with miR-499 AA genotype (adjusted OR = 0.74, 95%CI = 0.24-0.96). Subjects carrying miR-146a GG had a 0.62-fold (adjusted OR = 0.62, 95%CI = 0.33-1.20) decreased HCC risk, although no statistical significance was found. In patients infected with HBV, we did not find a statistical association of miR-146aG>C and miR-499A>G polymorphisms with HCC risk.

Table 3. Comparison of genotype frequencies and adjusted OR (95%CI) of miR-146aG>C and miR-499A>G polymorphisms.

Genotype	Controls	HCC patients	OR (95%CI)	P value	HBV patients	OR (95%CI)	P value
miR-146aG>C rs2910164							
CC	78	82	-		33	-	-
GC	71	62	0.83 (0.51-1.35)	0.36	25	0.86 (0.43-1.68)	0.53
GG	36	28	0.62 (0.33-1.20)	0.29	13	0.85 (0.36-1.91)	0.64
miR-499A>G rs3746444							
AA	123	128	-		54	-	-
AG	48	37	0.74 (0.24-0.96)	<0.05	14	0.65 (0.28-1.24)	0.23
GG	14	7	0.48 (0.16-1.15)	0.27	3	0.43 (0.09-1.85)	0.26

Adjusted for gender, age, smoking status, drinking status, and family history of cancer.

DISCUSSION

It is well known that genetic polymorphisms may be involved in the multistage hepatocarcinogenesis, and determine the susceptibility to the development of HCC (Akkiz et al., 2011a). The identification of SNPs that affect gene function or expression could contribute the susceptibility to HCC, and could play a predictive role in risk of cancer development and clarify the pathophysiologic mechanism of carcinogenesis. Therefore, identifying the genetic biomarkers of HCC susceptibility and their application in conjunction with traditional diagnosis, treatment and prognosis may contribute to lowering HCC mortality through early diagnosis, patient care and personalized therapy (Ludwig and Weinstein, 2005).

This molecular epidemiological study investigated whether the miR-146aG>C rs2910164 polymorphism could influence the susceptibility to the carcinogenesis of HCC. The polymorphisms of miR-146aG>C rs2910164 have an important role in various biological processes, including cell proliferation, immune response, cell differentiation and apoptosis, as well as tumorigenesis (Motsch et al., 2007; Bhaumik et al., 2008; Hurst et al., 2009; Pacifico et al., 2010). miR-146aG>C rs2910164 has a role as a pro-apoptotic molecule by inhibiting the nuclear factor kappa B(NFkB) pathway and blocking its impact on cell proliferation and metastasis of cancer cells (Bhaumik et al., 2008). A previous experimental study has indicated that loss of function of miR-146aG>C rs2910164 may enhance cell migration and invasion in vitro and that, on the contrary, increased expression could inhibit the cancer cell invasion (Bhaumik et al., 2008; Hurst et al., 2009). However, we did not find a significant association of variation of miR-146aG>C rs2910164 and risk of HCC in our study. The results of our study were similar to the findings of two previous studies conducted in Turkey (Akkiz et al., 2011a) and China (Xu et al., 2008). Akkiz et al. (2011a) reported that miR-146aG>C rs2910164 polymorphism has no role in genetic susceptibility to hepatocellular carcinogenesis. The study conducted in China showed no direct association of miR-146aG>C rs2910164 polymorphism with HCC in females. However, a 2-fold higher susceptibility to HCC was found in those male individuals with the CC genotype (Xu et al., 2008). The discrepancies of these results may be induced by different ethnicities, subject selection and sample size.

Our study demonstrated that the miR-499A>G rs3746444 polymorphisms are associated with decreased risk of HCC. A previous experimental study has indicated that miRNA-499 plays an important role in tumor biology and progression in various cancers, such as breast cancer, lung cancer, gallbladder and cervical squamous cell carcinoma (Liu et al., 2010;

Srivastava et al., 2010; Vinci et al., 2011; Zhou et al., 2011). There have been three studies on the association between miR-499A>G rs3746444 polymorphisms and HCC risk (Zhou et al., 2011; Akkiz et al., 2011b; Xiang et al., 2012). However, the results of these studies are inconsistent. Xiang reported that hsa-mir-499 polymorphism was associated with susceptibility to HBV-related HCC in a Chinese population (Xiang et al., 2012), while two studies conducted in China and Turkey did not find a significant association between miR-499A>G rs3746444 polymorphisms and HCC risk (Zhou et al., 2011; Akkiz et al., 2011b). Our study found that the miR-499A>G rs3746444 polymorphisms may alter the expression of mature miRNAs or their activities to target mRNA, and thus reduce cancer risk by variable mechanisms. Further large-sample studies are warranted to verify their association.

In conclusion, we did not find a significant association of miR-146aG>C rs2910164 and miR-499A>G rs3746444 polymorphisms with HCC risk in a Chinese population. Further investigations are warranted to clarify the relationship between miRNA polymorphisms and susceptibility to HCC risk in various ethnic populations.

ACKNOWLEDGMENTS

Research supported by the Zhejiang Natural Science Foundation (#Y12H030027), the Wenzhou Science and Technology Bureau (#Y20110078), the National Natural Science Foundation of China (#30700800, #81273282, #30972730), "973" Foundation (#2013CB531606), the Shanghai Municipal Commission for Science and Technology (11JC1410902), and Changhai Hospital (#CH125530300).

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