RESEARCH ARTICLE

Association Between Single Nucleotide Polymorphisms in miRNA196a-2 and miRNA146a and Susceptibility to Hepatocellular Carcinoma in a Chinese Population

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Abstract

Hepatocellular carcinoma (HCC) is one of the most prevalent cancers in the world and deeply threatens people's health, especially in China. Techniques of early diagnosis, prevention and prediction are still being discovered, among which the approaches based on single nucleotide polymorphisms in microRNA genes (miRNA SNPs) are newly proposed and show prospective potential. In particular, the association between SNPs in miRNA196a-2 (rs11614913) and miRNA146a (rs2910164) and HCC has been investigated. However, the conclusions made were conflicting, possibly due to insufficient sample size or population stratification. Further confirmations in well-designed large samples are still required. In this study, we verified the association between these two SNPs and the susceptibility to HCC by MassARRAY assay in a 2,000 large Chinese case-control sample. Significant association between rs11614913 and HCC was confirmed. Subjects with the genotype of CT+TT or T allele in rs11614913 were more resistant to HCC (CT+TT: OR (95% CI)=0.73 (0.57-0.92), *P*=0.01; T allele: OR (95% CI)=0.85 (0.75-0.97), *P*=0.02) and HBV-related HCC (CT+TT: OR (95% CI)=0.69 (0.53-0.90), *P*=0.01; T allele: OR (95% CI)=0.82 (0.71-0.95), *P*=0.01). The affected carriers of CT or TT also tended to have lower levels of serum AFP (*P*=0.01). This study demonstrated a role of rs11614913 in the etiology of HCC. Further research should focus on the clinical use of this miRNA SNP, so as to facilitate conquering HCC.

Keywords: Single nucleotide polymorphism in microRNA gene - HCC - MassARRAY

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Introduction

Hepatocellular carcinoma (HCC) is one of the most malignant cancers in the world, deeply threatening people's lives. Partially due to the high proportion of HBV infection in China, HCC (especially HBV-related HCC) is predominantly prevalent among the Chinese and widely affecting public health. (Jemal et al., 2011) Although therapeutic attempts can temporarily reduce the pain of patients, complete cure is still unavailable (de Lope et al., 2012). Therefore, early diagnosis and prevention is of essential importance, where more efforts are still required. Single nucleotide polymorphism (SNP), a well-known type of genetic marker, has become a prevalent biomarker in cancer research, as it is believed that SNPs in tumor related genes may alter gene expression and function thus change the susceptibility to cancers (Chung and Chanock, 2011). MicroRNA (miRNA) is a group of recently discovered molecules which has been proved to take effect by interacting with and regulating the target genes thus playing some roles in cancers (Kertesz et al., 2007; Bartels and Tsongalis, 2009). Therefore, as a novel sight, it is believed that SNPs in miRNA genes (also called miRNA SNPs), which may alter the expression, maturation and target recognition of miRNAs (Duan et al., 2007; Slaby et al., 2012), are a kind of expected biomarker of cancers. In particular, the SNPs in miRNA196a-2 (rs11614913) and miRNA146a (rs2910164) and their relationship to HCC have been studied (Wang et al., 2012; Han et al., 2013; Hu et al., 2013; Xu et al., 2013; Xu et al., 2013). However, the results were inconsistent with each other. The inconsistency mostly came from population stratification or small sample size with insufficient statistical power. Therefore, the conclusion whether these

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Table 1. General Characteristics of Hepatocellular Carcinoma Patients and Unaffected Controls

	Cases (n = 1000) No.(%) or mean ± SD	Controls (n = 1000) No.(%) or mean ± SD	*P*-value
Age (years)	54.7 ± 11.3	59.5 ± 11.6	<0.001
Gender			
Male	822 (82.2)	727 (72.7)	<0.001
Female	178 (17.8)	273 (27.3)	
Smoking status			
Never	667 (67.6)	528 (52.8)	<0.001
Ever	320 (32.4)	472 (47.2)	
Drinking status			
Never	736 (74.4)	737 (73.7)	0.72
Ever	253 (25.6)	263 (26.3)	
HBsAg (n = 954)			
Negative	181 (19.0)		
Positive	773 (81.0)		
Size of tumor foci (n = 542)			
<5cm	219 (40.0)		
≥5cm	323 (60.0)		
Number of tumor foci (n = 541)			
Single	477 (88.2)		
Multiple	64 (11.8)		
Tumor grade (n = 386)			
I—II	85 (22.0)		
III—IV	301 (78.0)		
Serum levels of tumor markers			
ALT (U/L, in 992 subjects)	58.4 ± 86.0		
AST (U/L, in 988 subjects)	62.2 ± 81.0		
AFP			
<20μg/L	364 (37.3)		
≥20μg/L	613 (62.7)		
(μg/L, in 405 subjects)	125.6 ± 288.3 (0-1210)		
HBV-DNA (n = 791)			
≥1000 IU/mL	457 (57.8)		
<1000 IU/mL	334 (42.2)		
(IU/mL, in 451 subjects)	$1.75\times10^6 \pm 5.43\times10^6$		

SNPs are associated with the susceptibility to HCC cannot be drawn definitely and requires further confirmations.

In this study, we verified the association between these two SNPs and the susceptibility to HCC in a 2000 Chinese case-control sample. We hope our efforts may get closer to the truth of the roles of miRNA SNPs in HCC and facilitate the use of miRNA SNPs in cancer prediction and prevention.

Materials and Methods

Subjects recruitment

The HCC patients confirmed by pathologic or imaging certification were collected from Huashan Hospital and Eastern Hepatobiliary Surgery Hospital. The unaffected control subjects without a history of cancer or other serious diseases were recruited randomly from the Taizhou Longitudinal Study. All subjects including both cases and controls were unrelated Chinese Han individuals residing in East China (Shanghai, Zhejiang, Jiangsu and Jiangxi). At enrolment, health and life style questionnaires, medication usage and family history data were collected by a trained interviewer. For HCC patients, clinical indexes were also collected, such as serum levels of AFP, HBsAg, HBV-DNA, ALT, AST and total bilirubin, the number, size and grade of tumor foci. The whole procedure was approved by local ethic review committee.

DNA extraction and genotyping

Genomic DNA of every subject was isolated from peripheral blood by using AxyPrep™ Blood Genomic DNA Miniprep Kit (Axygen Biosciences, Union City, United States). The genotyping of rs11614913) and rs2910164 was achieved by Sequenom MassARRAY technique (Oeth et al., 2009). The primers were designed by MassARRAY Assay Design Software and synthetized by Invitrogen Corporation Shanghai Representative Office. Sequencing was performed on MassARRAY Compact System (SEQUENOM Corporation) and the data were analyzed by TYPER Analyzer software 4.0.

Statistical analysis

Hardy-Weinberg Equilibrium (HWE) of the genotypes was tested by Pearson chi-square test of goodness-of-fit, which was carried out using the software Excel 2000 (Microsoft, Redmond, United States). Binary logistic regression was used to estimate the association between the genotypes/allele types of miRNA SNPs and the susceptibility to HCC/HBV-related HCC after adjusting the confounding factors. Pearson chi-square test of independence was then used to investigate the association between the SNPs and qualitative clinical indexes in HCC patients. For quantitative indexes, which had heterogeneity of variance or non-normal distributions, analysis of variance or nonparametric tests were applied respectively. These statistical tests were carried out using the software SPSS 13.0 for Windows (SPSS, Chicago, United States). The statistical power of the association tests between the SNPs and susceptibility to HCC was evaluated by QUANTO software.

Results

Sample overview

A total of 2000 subjects were enrolled in this study, including 1000 HCC patients (cases) and 1000 unaffected controls. Among the HCC patients, 773 subjects positive with HBsAg were treated as HBV-related HCC cases. General characteristics of all subjects were listed in Table 1. It was shown that age, gender and smoking status were significantly different between the cases and the controls (controls older than cases), while drinking status was not discrepant between the two groups.

The miRNA SNPs investigated in this study (rs11614913 and rs2910164) was listed in Table 2. 1991 and 1995 subjects were successfully genotyped for rs11614913 and rs2910164, respectively. Pearson's chi-square tests of goodness-of-fit showed that the genotypes of both SNPs followed HWE in cases and controls.

Association between the miRNA SNPs and susceptibility to hepatocellular carcinoma

We observed statistically significant association between rs11614913 in miRNA196a-2 and the susceptibility to HCC, after the adjustment of confounding factors such as sex, age, smoking and drinking habit

Table 2. The miRNA SNPs Investigated in This Study

SNP ID	Substitution	miRNA	Chromosome start-stop site	SNP Location	Amplification primers	Extension primer
rs11614913	C/T	hsa-mir-196a-2	chr12:54385522 -54385631	54385599 (mat)	ACGTTGGATGTCGACGAAAACCGACTGATG ACGTTGGATGGGTAGTTTCATGTTGTTGGG	TCGGCAACA AGAAACTG
rs2910164	C/G	hsa-mir-146a	chr5:159912359 -159912457	159912418 (min)	ACGTTGGATGCCACGATGACAGAGATATCC ACGTTGGATGGAACTGAATTCCATGGGTTG	TGTCAGTGT CAGACCT

Table 3. Association Between the miRNA SNPs and Susceptibility to HCC

Genotypes/	Controls	HCC patients			HBV-related HCC patients		
Allele types	no. (%)	no. (%)	OR(95% CI)†	*P*-value†	no.(%)	OR(95% CI)†	*P*-value†
rs11614913	n =995	n =996			n =771		
CC	165 (16.6)	214 (21.5)	1.00		171 (22.2)	1.00	
CT	502 (50.4)	488 (49.0)	0.70 (0.53-0.92)	0.01*	376 (48.8)	0.64 (0.48-0.87)	0.00*
TT	328 (33.0)	294 (29.5)	0.75 (0.58-0.96)	0.02*	224 (29.0)	0.72 (0.55-0.95)	0.02*
Dominant model (CC vs. CT+TT)			0.73 (0.57-0.92)	0.01*		0.69 (0.53-0.90)	0.01*
Recessive model (CC+CT vs.TT)			1.16 (0.95-1.42)	0.15		0.82 (0.65-1.02)	0.07
C	832 (41.8)	916 (46.0)	1.00		717 (46.6)	1.00	
T	1158 (58.2)	1076 (54.0)	0.85 (0.75 -0.97)	0.02*	823 (53.4)	0.82 (0.71-0.95)	0.01*
rs2910164	n =998	n =997			n =771		
GG	156 (15.6)	163 (16.3)	1.00		124 (16.1)	1.00	
CG	475 (47.6)	503 (50.5)	1.18 (0.96-1.45)	0.12	390 (50.6)	1.20 (0.95-1.50)	0.12
CC	367 (36.8)	331 (33.2)	1.18 (0.89-1.57)	0.25	257 (33.3)	1.15 (0.84-1.56)	0.39
Dominant model (GG vs. CG+CC)			1.07 (0.83-1.38)	0.59		0.97 (0.73-1.28)	0.82
Recessive model (GG+CG vs.CC)			1.18 (0.97-1.44)	0.10		0.85 (0.68-1.05)	0.12
G	787 (39.4)	829 (41.6)	1.00		638 (41.4)	1.00	
C	1209 (60.6)	1165 (58.4)	1.11 (0.97 -1.27)	0.12	902 (58.6)	0.91 (0.78-1.05)	0.20

†ORs and *P*-values were all obtained after adjustment of age, gender, smoking status and drinking status; **P*-value less than 0.05

Table 4. Association Between the miRNA SNPs and Clinical Indexes in HCC Patients

Indexes	Genotype			*P*-value
AFP (μg/L)				
rs11614913	CC	CT	TT	
	168.3±27.1	100.5±15.0	135.7±36.2	0.01*

**P*-value less than 0.05

using binary logistic regression. As shown in Table 3, the genotype of CT+TT or T allele was significantly associated with decreased risk of HCC (CT+TT: OR (95% CI)=0.73 (0.57-0.92), *P*=0.01; T allele: OR (95% CI)=0.85 (0.75-0.97), *P*=0.02), as compared with CC genotype or C allele. The association between CT+TT or T allele and decreased risk of HBV-related HCC was also revealed (CT+TT: OR (95% CI)=0.69 (0.53-0.90), *P*=0.01; T allele: OR (95% CI)=0.82 (0.71-0.95), *P*=0.01). These findings indicated that rs11614913 may play some roles in the etiology of HCC and HBV-related HCC. In addition, it was found that the best fit genetic model of the significant association was dominant model, suggesting that subjects with T alleles are more unlikely to be affected by HCC or HBV-related HCC.

We also examined the association between rs2910164 in miRNA146a and the susceptibility to HCC/HBV-related HCC (Table 3). The results were not statistically significant, indicating that rs2910164 may not be associated with the risk of HCC or HBV-related HCC. All of these association tests were above 80% statistical power to detect the OR of at least 1.5.

Association between the miRNA SNPs and clinical indexes in HCC patients

It was well documented that clinical indexes, such as AFP, total bilirubin, ALT, AST and HBV-DNA, could be used to evaluate the progression as well as prognosis of HCC (Zhou et al., 2012). In this study, rs11614913 was observed to be significantly associated with serum level of AFP (*P*=0.01, Table 4). Compared to CC genotype, subjects with CT or TT genotype tended to have lower AFP level. As AFP was a widely used diagnosis indicator of HCC (Johnson, 2001; Lee et al., 2013), this result was quite in accordance to the findings above that CT+TT or T allele was correlated to lower risk of HCC, thus also further confirming the role of rs11614913 in HCC.

There was no significant association observed between the SNPs and other clinical indexes including total bilirubin, HBV-DNA, ALT, AST and the number, size and grade of tumor foci (Data not shown).

Discussion

The early diagnosis and prevention of cancers is a world-wide problem. As to HCC, it is much more critical in China because of the high prevalence of HBV infection which is a key inducer of HCC (Iavarone and Colombo, 2013). Although researchers have been working on this problem for a long time and some positive results have been reported, the clinical use is not yet within the foreseeable future (Sengupta and Siddiqi, 2012; Wang et al., 2013). In this case, more efforts to search for new biomarkers and detecting techniques are still summoned.

MiRNA is thought to be such a kind of new and promising biomarker. It can interact with target genes by direct binding based on complementary pairing or secondary structure, thus affecting the expression and/or function of target genes, which may take some parts in the pathology of HCC (Wong et al., 2013).

Indeed, the expression profile of multiple miRNAs has been investigated and some hopeful panels have been documented (Zhou et al., 2011; Zhang et al., 2013), which may promote the early diagnosis, prediction and prevention of HCC, although more clinical confirmations are still required.

Recently, SNPs in the genomic region of miRNAs (miRNA SNPs) have come into sight. As its detection is mostly based on leukocytes in peripheral blood, the process is flexible and the results are reliable. In addition, as the SNPs are germline variations, cancer prediction and prevention made based on them should be much earlier in lifetime, as compared to non-congenital and somatic biomarkers.

The C>T substitution polymorphism in miRNA196a-2 (rs11614913) is one of the most frequently studied miRNA SNPs and has been reported to be associated with several types of cancer, such as non-small-cell lung cancer (Hu et al., 2008), breast cancer (Hoffman et al., 2009), gastric cancer (Peng et al., 2010) and oral carcinomas (Liu et al., 2012). Previous studies have also investigated its relationship to the susceptibility to HCC but conflicting results were displayed, even between meta-analyses (Wang et al., 2012; Xu et al., 2013). Although the total sample sizes were large in meta-analyses, it was shown that most of the studies enrolled in the meta-analyses were done on small samples. Therefore, drift between significant and insignificant association is understandable, because of bias generated from sample selection and stratification of individual studies as well as the whole meta-analysis. A recent original research failed to reveal the association between rs11614913 and the susceptibility to HCC, in a 1021 vs. 1012 case-control sample collected from North, East and West China (Han et al., 2013). In this study with a 1000 vs. 1000 sample from East China, we revealed that as compared with wide type CC genotype, CT/TT genotype of rs11614913 had a less risk of susceptibility to HCC.

It was well documented that miRNA196a-2 was over-expressed in a wide variety of malignancies (Liu et al., 2012). The substitution from C to T of rs11614913 could alter miRNA processing and lead to the reduction of mature miRNA196a-2 (Ryan et al., 2010). It was proven that higher expression level of miRNA196a-2 was significantly correlated with CC genotype or at least one C allele than TT genotype (Li et al., 2010). Furthermore, homeobox (HOX) gene cluster and annexinA1 (ANXA1) gene were confirmed as targets of miRNA196a-2 (Yekta et al., 2004; Luthra et al., 2008). The deregulation of these target genes because of high expression of miRNA196a-2 could lead to carcinogenesis and malignant transformation of HCC (Akkiz et al., 2011). These known knowledge may explain the mechanism why those with CC genotype of rs11614913 tended to suffer from HCC. As a result, we revealed in this study that subjects with CC genotype were more likely to have higher level of serum AFP, which was an important indicator of HCC. Thus, taken these together, it is believed that rs11614913 in miRNA196a-2 should take some roles in the pathology of HCC.

The G>C substitution polymorphism in miRNA146a (rs2910164) has also been studied whether it is associated with HCC. Unfortunately, because of the same reason as the studies of rs11614913, the results were inconsistent with each other either (Wang et al., 2012; Hu et al., 2013; Xu et al., 2013; Xu et al., 2013). In this large sample based study, we failed to find any significant association between rs2910164 and the susceptibility to HCC, indicating that rs2910164 may not take any part in the etiology of HCC.

In conclusion, by this large sample based association study of miRNA SNPs and HCC, it is concluded that rs11614913 in miRNA196a-2 but not rs2910164 in miRNA146a was associated with the susceptibility to HCC, which may help to the developing of early prediction and prevention strategies for HCC. Further researches on this miRNA SNP are highly recommended so as to facilitate the clinical use of the achievement gained from basic research.

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