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Association between polymorphisms in the promoter region of pri-miR-34b/c and risk of hepatocellular carcinoma

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ABSTRACT. Hepatocellular carcinoma (HCC) is a major cause of cancer-related deaths worldwide. MicroRNA-34 (*miR-34*) gene plays a key role in altering the apoptotic cycle and pathways of downstream

cells, and therefore influences carcinogenesis. In this case-control study, we assessed the role of the pri-miR-34b/c rs4938723 polymorphism in HCC risk. The pri-miR-34b/c polymorphic genotype was determined in 286 patients with HCC and 572 controls using polymerase chain reaction-restriction fragment length polymorphism. The male gender (χ^2 = 12.95, P < 0.001), regular alcohol consumption (χ^2 = 16.81, P < 0.001), and a family history of cancer (χ^2 = 11.88, P = 0.001) were associated with HCC risk. However, the age (t = 1.19, P = 0.12) and tobacco smoking habit (χ^2 = 0.64, P = 0.42) of HCC patients were comparable to those of the controls. The TC (adjusted OR = 1.46, 95%CI = 1.06-2.01) and CC (adjusted OR = 3.07, 95%CI = 1.77-5.34) genotypes of pri-miR-34b/c rs4938723 were correlated with a higher risk of HCC compared to the TT genotype. Moreover, the TC+CC genotype was correlated with an increased risk of HCC compared to the TT genotype (adjusted OR = 1.64, 95%CI = 1.21-2.22). In the recessive model, the CC genotype of pri-miR-34b/c rs4938723 was significantly correlated with an elevated risk of HCC compared to the TT+TC genotype (adjusted OR = 2.50, 95%CI = 1.49-4.22). Further large-scale and multi-center studies are required to confirm these results.

Key words: pri-miR-34b/c; Polymorphism; Hepatocellular carcinoma

INTRODUCTION

Hepatocellular carcinoma (HCC) is a major cause of cancer-related deaths worldwide (IARC, 2012). The etiology of HCC has been widely studied; however, its actual mechanism is still unclear. The pathogenesis of HCC is influenced by a number of environmental factors, such as infection with hepatitis B virus and hepatitis C virus, and lifestyle factors, such as long-term alcohol consumption, as well as contaminated water and aflatoxin consumption (Chitapanarux and Phornphutkul, 2015; de Martel et al., 2015; Kikuchi et al., 2015; Kubo et al., 2015). Therefore, hereditary factors may contribute to the development of HCC. Moreover, familial aggregation and co-aggregation of HCC have indicated that genetic factors play a major role in the development of HCC. Previous molecular studies have reported that genetic factors, such as the genes coding for human leukocyte antigen (HLA)-DRB1, FasL, epidermal growth factor, human serum antigen-miR-1269, cyclooxygenase-2, and methylene tetrahydrofolate reductase, play an essential role in HCC susceptibility (Shen et al., 2015; Wang et al., 2015; Xiong et al., 2015; Zhang et al., 2015; Khalifa et al., 2016; Ma et al., 2016).

Previous studies have revealed that miRNA contributes to the initiation, progression, metastasis, and drug resistance of HCC (Li et al., 2015). Changes in microRNA-34 (*miR-34*) expression could influence the expression of their downstream genes, thereby influencing carcinogenesis. *miR-34b* and *miR-34c* are homologous to *miR-34* (Guo et al., 2012). CpG is present upstream of the transcription start site of the pri-miR-34b/c CpG island (Guo et al., 2012). CpG island methylation has been reported to inhibit the activation of miR-34 via p53, thereby influencing cell proliferation and increasing the risk of carcinogenesis (Xu et al., 2011; Wang et al., 2014; Zhang et al., 2014b). So far, three studies have investigated the association

between polymorphisms in the promoter region of *miR-34b/c* and HCC risk; however, the results of these studies are inconsistent (Xu et al., 2011; Han et al., 2013; Son et al., 2013). Moreover, these studies did not analyze the interaction between the *pri-miR-34b/c* rs4938723 polymorphism and environmental factors in HCC risk. In this 1:2 matched case-control study, we have evaluated the possible role of the *pri-miR-34b/c* rs4938723 polymorphism in HCC risk, and its interaction with environmental factors in a Chinese population.

MATERIAL AND METHODS

Patients

A total of 286 patients with HCC were enrolled from the First People's Hospital of Wenling and Zhongshan Hospital of Shanghai between March 2013 and October 2015. HCC was confirmed in all patients via pathological examination. HCC patients with autoimmune hepatitis, drug-induced hepatitis, Wilson's disease, HIV infection, syphilis, or secondary or metastatic HCC were excluded from this study. A total of 572 controls were recruited from the Outpatient Clinics of our hospital and health examination center between March 2013 and October 2015. Individuals without a history of chronic or acute infectious disease, tumors, or end-stage kidney disease were included in this study.

The demographic and clinical variables of all HCC patients and control subjects were collected by a standard questionnaire or medical records. The clinical variables (TNM stage and Child-Pugh score) were collected from the patient medical records. Written informed consent was obtained from all patients with HCC and control subjects prior to the study. The study protocol was approved by the Ethics Committee of the First People's Hospital of Wenling.

Genotyping

Venous blood samples (5 mL) were obtained from all enrolled patients with HCC and control subjects. DNA was extracted from the venous blood samples using a Tiangen Blood DNA kit (Tiangen, Beijing, China). The *pri-miR-34b/c* rs4938723 polymorphism was genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) using the primers (5'-CTCTGGGAACCTTCTTTGACCCAT-3' and 5'-TGAGATCAAGGCC ATACCATTCAAGA-3') designed by Sangon Biological Engineering Ltd. (Shanghai, China). The PCR products were digested using the *Bcc*I restriction enzyme (Sangon Biological Engineering Ltd.). The thermal cycling program for porous PCR (MJ Research Inc., Waltham, MA, USA) was as follows: initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 45 s, annealing at 63°C for 45 s, and extension at 72°C for 45 s; and a final extension at 72°C for 5 min. For quality control, 10% of the samples were randomly genotyped again and sequenced to validate the RFLP findings.

Statistical analysis

The demographic, lifestyle, and clinical variables were compared between the case and control groups by the chi-square test or student *t*-test. The genotype frequencies of the *pri-miR-34b/c* rs4938723 polymorphism were compared between the HCC patients and controls

using the chi-square test. Deviation of the HCC patients and control individuals from the Hardy-Weinberg equilibrium (HWE) was calculated by the chi-square test. The odds ratios (ORs) and 95% confidence intervals (95%CI) associated with HCC risk were determined by logistic regression analysis, taking the control individuals as the reference group. The association between the *pri-miR-34b/c* rs4938723 polymorphism and risk of HCC were analyzed by the co-dominant, dominant, and recessive models. All statistical analyses were performed using the SPSS v.20.0 software package (SPSS Inc., Chicago, IL, USA). The differences were considered to be statistically significant at P values < 0.05.

RESULTS

The demographic and clinical variables of investigated subjects are summarized in Table 1. The mean ages of HCC patients and controls were 61.42 ± 10.67 and 60.45 ± 11.47 years, respectively. The male gender (χ^2 = 12.95, P < 0.001), regular alcohol consumption (χ^2 = 16.81, P < 0.001), and a family history of cancer (χ^2 = 11.88, P = 0.001) were associated with HCC risk. However, the age (*t* = 1.19, P = 0.12) and tobacco smoking habit (χ^2 = 0.64, P = 0.42) of HCC patients were comparable to those of the controls. Ninety-eight of the included HCC patients (34.27%) presented TNM stage I-II, and the remaining 188 (65.73%) presented TNM stage III-IV. Sixty-one (21.33%), 121 (42.41%), and 104 (36.36%) of the cases presented a Child-Pugh score of A, B, and C, respectively.

Table 1. Demographic and clinical variables of included patients and controls.

Variables	Patients (N = 286)	%	Controls (N = 572)	%	χ^2 test or *t*-test	P value
Age (years)	61.42 ± 10.67		60.45 ± 11.47		1.19	0.12
Gender						
Females	204	71.33	336	58.74		
Males	82	28.67	236	41.26	12.95	<0.001
Tobacco smoking						
Never	176	61.54	368	64.34		
Ever	110	38.46	204	35.66	0.64	0.42
Alcohol consumption						
Never	136	47.55	356	62.24		
Ever	150	52.45	216	37.76	16.81	<0.001
Family history of cancer						
No	264	92.31	557	97.38		
Yes	22	7.69	15	2.62	11.88	0.001
TNM stage						
I-II	98	34.27				
III-IV	188	65.73				
Child-Pugh classification						
A	61	21.33				
B	121	42.31				
C	104	36.36				

The genotype frequencies of the *pri-miR-34b/c* rs4938723 polymorphism in HCC patients and controls are summarized in Table 2; 102 (35.66%), 146 (51.05%), and 38 (13.29%) patients with HCC and 272 (47.55%), 267 (46.68%), and 33 (5.77%) control subjects expressed the TT, TC, and CC genotypes, respectively. We observed significant differences in the genotype frequencies of the *pri-miR-34b/c* rs4938723 polymorphism between HCC patients and controls (χ^2 = 19.96, P < 0.001). The distributions of the *pri-miR-34b/c* rs4938723 TT, TC, and CC genotypes conformed to the HWE in patients, and deviated from the HWE in controls.

Table 2. Genotype distributions of the *pri-miR-34b/c* rs4938723 polymorphism in hepatocellular carcinoma (HCC) patients and controls.

Genotypes	Patients (N = 286)	%	Controls (N = 572)	%	χ^2 test	P value	HWE for patients		HWE for controls	
							χ^2 test	P value	χ^2 test	P value
TT	102	35.66	272	47.55						
TC	146	51.05	267	46.68						
CC	38	13.29	33	5.77	19.96	<0.001	1.60	0.21	75.81	< 0.001

HWE, Hardy-Weinberg equilibrium.

We then analyzed the association between the *pri-miR-34b/c* polymorphism and HCC risk (Table 3). Conditional logistic regression analysis indicated that the TC and CC genotypes of *pri-miR-34b/c* rs4938723 were correlated with a higher risk of HCC compared to the TT genotype (TC genotype: adjusted OR = 1.46, 95%CI = 1.06-2.01 and CC genotype: adjusted OR = 3.07, 95%CI = 1.77-5.34). Moreover, the TC+CC genotype of *pri-miR-34b/c* rs4938723 was correlated with an increased risk of HCC compared to the TT genotype (adjusted OR = 1.64, 95%CI = 1.21-2.22). In the recessive model, the CC genotype of *pri-miR-34b/c* rs4938723 was significantly correlated with an elevated risk of HCC, compared to the TT+TC genotype (adjusted OR = 2.50, 95%CI = 1.49-4.22).

Table 3. Association between the *pri-miR-34b/c* rs4938723 polymorphism and risk of hepatocellular carcinoma (HCC).

Genotypes	Patients (N = 286)	%	Controls (N = 572)	%	Adjusted OR (95%CI)[1]	P value
Co-dominant						
TT	102	35.66	272	47.55	1.0 (Ref.)	-
TC	146	51.05	267	46.68	1.46 (1.06-2.01)	0.01
CC	38	13.29	33	5.77	3.07 (1.77-5.34)	< 0.001
Dominant						
TT	102	35.66	272	47.55	1.0 (Ref.)	-
TC+CC	184	64.34	300	52.45	1.64 (1.21-2.22)	0.001
Recessive						
TT+TC	248	86.71	539	94.23	1.0 (Ref.)	-
CC	38	13.29	33	5.77	2.50 (1.49-4.22)	< 0.001

[1]Adjusted for age, gender, tobacco smoking, alcohol consumption, and family history of cancer. OR, odds ratio; CI, confidence interval.

DISCUSSION

Recently, genomic susceptibility to diseases has attracted a considerable amount of attention. In this study, we observed that individuals harboring the TC and CC genotypes of pri-miR-34b/c were at a higher risk of HCC than those expressing the TT genotype.

The *pri-miR-34b/c* rs4938723 polymorphism is known to be located at the core promoter region of *pri-miR-34b/c*. Changes in the expression of *miR-34* could influence the expression of the downstream gene, as *miR-34* is a key gene regulating the cell cycle and apoptotic pathways of downstream cells. The rs4938723 polymorphism has been shown to influence the cell cycle of downstream cells, as well as induce changes in the expression of key genes in the apoptotic pathway, thereby influencing cancer susceptibility. Several studies have reported a significant association between the rs4938723 polymorphism and risk of malignant tumors such as acute lymphoblastic leukemia, cervical cancer, papillary thyroid carcinoma,

gastric cancer, esophageal squamous cell carcinoma, renal cell cancer, and colorectal cancer (Oh et al., 2014; Zhang et al., 2014a,b; Chen et al., 2015; Pan et al., 2015; Tong et al., 2016; Yuan et al., 2016). A previous large-scale case-control study comprising 1109 esophageal squamous cell carcinoma patients and 1275 healthy subjects reported a correlation between the GG genotype of miR-34b/c and a significantly reduced risk of esophageal squamous cell carcinoma in a Chinese population (Zhang et al., 2014a). Additionally, the C allele of *pri-miR-34b/c* rs4938723 was shown to be associated with an increased risk of renal cell cancer (RCC) in a Chinese population in a case-control study comprising 710 RCC patients and 760 controls (Zhang et al., 2014b). Tong et al. (2016) revealed a correlation between the *pri-miR-34b/c* polymorphisms and risk of developing childhood acute lymphoblastic leukemia by influencing the transcription activity of the *pri-miR-34b/c* promoter in Chinese acute lymphoblastic leukemia patients. On the other hand, Yuan et al. (2016) reported that the *pri-miR-34b/c* rs4938723 polymorphism could play an important role in the pathogenesis of cervical cancer, based on the results of a hospital based case-control study with 328 cervical cancer patients and 568 healthy subjects. Chen et al. (2015), who analyzed 784 patients with papillary thyroid carcinoma and 1006 healthy controls, reported a correlation between the *pri-miR-34b/c* rs4938723 polymorphism and the risk of developing papillary thyroid carcinoma. Pan et al. (2015), in a study with 197 gastric cancer patients and 289 controls, reported the possible role of *pri-miR-34b/c* rs4938723 in gastric cancer susceptibility. Finally, Oh et al. (2014) reported that *pri-miR-34b/c* polymorphisms were associated with a reduced risk of colorectal cancer, based on the results of a study conducted in 545 colorectal cancer patients and 428 healthy controls. So far, three previous studies have reported the relationship between *pri-miR-34b/c* polymorphisms and risk of HCC with inconsistent results (Xu et al., 2011; Han et al., 2013; Son et al., 2013). Xu et al. (2011) reported a positive correlation between the TC and CC genotypes of *pri-miR-34b/c* and HCC risk in 501 HCC patients and 548 cancer-free controls. Han et al. (2013) observed a significant positive association between the CC genotype of *pri-miR-34b/c* rs4938723 and HCC risk in a large-scale study conducted in a Chinese population. Additionally, in a study conducted in 157 HCC patients and 201 cancer-free control subjects, Son et al. (2013) discovered that the loss of the T allele in *pri-miR-34b/c* could increase the risk of HCC in a Korean population. In our study, we observed a positive correlation between the *pri-miR-34b/c* polymorphism and risk of HCC in a Chinese population in all genetic models.

The results of our study are subject to two limitations. Firstly, the HCC patients and control subjects were selected from two hospitals; therefore, the sample population did not represent the overall population. Secondly, other genetic polymorphisms in miRNA may interact with *pri-miR-34b/c*, which may induce bias in our study. In conclusion, we discovered that the *pri-miR-34b/c* rs4938723 polymorphism is positively correlated with HCC development in the Chinese population of east coastal areas.

Conflicts of interest

The authors declare no conflicts of interest.

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