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A potentially functional polymorphism in the promoter region of *miR-34b/c* is associated with renal cell cancer risk in a Chinese population

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Members of the miR-34 family have been shown to be transcriptional targets of the tumour suppressor gene *P53*. Aberration expression of miR-34 impairs p53-mediated cell cycle arrest and apoptosis. A single nucleotide polymorphism T > C (rs4938723) located within the CpG island in the promoter region of *pri-miR-34b/c* may affect its expression and has been suggested to influence cancer risk. In this study, we genotyped rs4938723 using the TaqMan method to explore the relationship between this polymorphism and the risk of renal cell cancer (RCC) in a case-control study of 710 RCC patients and 760 control subjects. We found that individuals carrying the CC genotype had a significantly increased RCC risk compared with those with TT or TT/TC genotypes [odds ratio (OR) = 1.53, 95% confidence interval (CI) = 1.06–2.21 for CC vs. TT and OR = 1.48, 95% CI = 1.05–2.10 for CC vs. TT/TC). Furthermore, the increased risk was more evident in the subgroups of older subjects (OR = 1.80, 95% CI = 1.08–3.01), males (OR = 1.64, 95% CI = 1.08–2.51), smokers (OR = 2.07, 95% CI = 1.16–3.69) and drinkers (OR = 1.94, 95% CI = 1.01–3.73), although no interaction between rs4938723 and these characteristics was observed. Twenty-seven normal tissues adjacent to tumour were used to evaluate the association between the expression level of miR-34b/c and the polymorphism, which revealed higher expression levels of miR-34b/c in normal renal tissues with TT+TC genotypes than in those with CC genotypes ($P < 0.01$). Furthermore, a luciferase gene assay in 293-T cells showed that the luciferase activities with rs4938723 T allele are higher than that with C allele ($P < 0.05$). These results suggest that the *miR-34b/c* rs4938723 C allele may increase susceptibility to RCC by decreasing the activity of *pri-miR-34b/c* promoter.

Introduction

Renal cell cancer (RCC) is the most common malignancy of the kidney (>80%) and the 10th most common cause of cancer-related deaths among men (1,2). The distribution of RCC varies around the world, with the highest incidence in the developed countries, and its incidence is increasing (1). It is estimated that 37.7 men and 16.6 women per 100 000 Chinese people are diagnosed with RCC every year (3). The exact causes of RCC remain unknown, but evidence is accumulating to suggest a strong connection between the development of RCC and several risk factors, such as cigarette smoking, obesity, a history of hypertension and occupational factors such as exposure to asbestos, petroleum derivatives, trichloroethylene, pesticides, polycyclic hydrocarbons and other compounds (4,5).

MicroRNAs (miRNAs) are a class of small non-coding RNA molecules composed of ~22 nucleotides. They act as post-transcriptional regulators that bind to complementary sequences in the 3′ untranslated regions of target messenger RNA transcripts, leading to translational repression and gene silencing (6,7). *TP53* tumour suppressor gene mutations have been found in nearly all types of cancers, including RCC (8,9). It has been suggested that p53 can regulate the expression of miRNAs, especially the miR-34 family members. The miR-34 family contains three miRNAs: miRNA-34a, which is encoded by its own transcript, and miRNA-34b and miRNA-34c, which share one common primary transcript (10,11). It has been shown that the miR-34 family is a part of the p53 network and the expression of them is directly induced by p53 in response to DNA damaging agents or activation of oncogenes (10,12). The promoter regions of the miR-34 family contain CpG islands, and Vogt *et al.* (13) suggested that miR-34 inactivation is a common event in tumour formation, including RCC, and that the CpG methylation of miR-34a and miR-34b/c may have diagnostic value. It is also indicated in research by Lodygin *et al.* (14) that the expression of miR-34a is silenced in several cancers including kidney cancers.

Accumulating evidence suggests that single nucleotide polymorphisms (SNPs) in human miRNA genes can alter the expression of miRNA and affect hundreds of target genes (15). Recently, Xu *et al.* (16) showed that the rs4938723 (T>C) polymorphism, which is located in the typical CpG island in the promoter region of *pre-miR-34b/c*, was associated with an increased risk of primary hepatocellular carcinoma. Son *et al.* (17) obtained the same results; that the T allele in *miR-34b/c* T>C (rs4938723) decreases the risk of hepatocellular carcinoma in the Korean population (17). In addition, Gao *et al.* (18) suggested that the rs4938723 in the promoter region of pri-miR-34b/c plays a protective role in the development of colorectal cancer. The web-based SNP analysis tool TFSEARCH 1.3 suggested that the T to C base change could have an effect on predicted GATA-X transcription factors binding. Considering that miR-34 is frequently inactivated in RCC (13), we hypothesised that rs4938723 polymorphism could also have impacts on the development of RCC. To test this hypothesis, we genotyped this SNP using the TaqMan method and assessed its associations with risk of RCC in a case-control study of 710 RCC cases and 710 age- and sex-matched cancer-free controls in a Chinese population. Furthermore, to explore the possible mechanism, we also performed real-time polymerase chain reaction (RT-PCR) and luciferase reporter gene assays.

Materials and methods

Study subjects

This is an ongoing molecular epidemiological study of RCC conducted in the First Affiliated Hospital of Nanjing Medical University, Nanjing, China, from May 2004. The study was approved by the institutional review board of the Nanjing Medical University. In total, 710 RCC cases and 760 cancer-free controls were recruited. The design of the study and the inclusion criteria of the subjects have been described previously (19). In brief, all subjects in our study are ethnic Han Chinese coming from different families without blood relationship. They are recruited from the same region of China. All the patients were newly diagnosed and histopathologically confirmed incident RCC cases without history of other cancers or previous chemotherapy or radiotherapy, and they were consecutively recruited without restriction by age and sex. The controls were recruited from healthy subjects who were seeking physical examination in the outpatient departments at the hospital and were frequency matched to the cases on age (±5 years) and sex. Compared with our previously published studies (19), an additional 90 cases and 137 controls, recruited recently, were added to this study. At recruitment, written informed consent was obtained from all participants involved in this study.

DNA extraction and genotyping

Genomic DNA was isolated and purified from peripheral blood by proteinase K digestion and phenol/chloroform extraction. The SNP rs4938723 was genotyped using predesigned TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA). The sequences of the primer and probe for each SNP were available on request. According to the manufacturer's instructions, amplifications were performed in the 384-well ABI 7900HT Real-Time PCR System (Applied Biosystems). After completing the amplification, SDS 2.3 automated software was used to read and analyse the fluorescence intensity in each well of the plate. The negative and positive controls were included in each plate to ensure accuracy of the genotyping. In addition, ~10% of samples were randomly selected for repeated assays, and the results were all concordant.

Analysis of hsa-miR-34b/c expression

To further evaluate correlation between the expression of miR-34b/c and rs4938723 polymorphism, 27 normal tumour-adjacent renal tissues from patients with different genotypes were collected to perform RT-PCR. Total RNA from these tissues were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. All samples were reverse transcribed by the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) and 5× specific TaqMan microRNA assays (Applied Biosystems). ABI 7900HT Real-Time PCR system was used to perform quantitative PCR by TaqMan Universal PCR Master Mix (Applied Biosystems) and 20× specific TaqMan microRNA assays (Applied Biosystems). The reaction was performed by 7900HT Real-Time PCR system (Applied Biosystems) and with the following conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. All reactions were conducted in triplicate and normalised on the basis of the expression levels of U6.

Construction of reporter plasmids

The association between the polymorphism and the expression of miR-34b/c was further explored *in vivo*. The 1001-bp fragment in promoter region containing wild-type of rs4938723 T was synthesised chemically (by Invitrogen) and mutated by using the primers of 5′-CTCCCTTCTATTTCTGATACAAA TAGTTTCTACTATTC-3′ (forward) and 5′-GTCCTCAATGAGAGCTGTG ATAGGTCAAAGAAGGTTCCCAGAG-3′ (reverse). By the restriction sites of MluI and XhoI, the wild-type and variant fragments were cloned into the pGL3-basic vector (Promega, Madison, WI, USA) containing firefly luciferase gene as a reporter. Finally, both constructs were confirmed by DNA sequencing, and no errors were found.

Transfection and luciferase reporter assays

The cell line of 293-T was seeded into 24-well culture plates. After 24 h, each well was co-transfected with 800 ng of promoter luciferase plasmid and 0.8 ng of pRL-SV40 (Promega) containing Renilla luciferase gene as an internal standard. Additionally, the pGL3-basic vector was used as a negative control. The cells were collected and lysed by passive lysis buffer (Promega) 48 h after transfection, and the relative luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Transfections were conducted in independent triplicate experiments for each plasmid construct.

Statistical analysis

SNP allele frequencies were tested against departure from Hardy–Weinberg equilibrium using a goodness-of-fit χ^2 test before analysis. Differences in demographic characteristics, selected variables and frequencies of the genotypes of the SNP between the cases and controls were evaluated by using the χ^2 test (for categorical variables) and Student's *t*-test (for continuous variables). Associations between the genotypes and risk of RCC were estimated by computing odds ratios (ORs) and 95% confidence intervals (CIs) from logistic regression analysis with adjustment for age, sex, body mass index (BMI), smoking years, drinking status, hypertension and diabetes. The relative expression levels of miR-34b/c in all samples were calculated by the $2^{-\Delta ct}$ method compared with the levels of U6. The unpaired Student's *t*-test was used to evaluate the associations between the expression of miR-34b/c and rs4938723 polymorphism and differences in dual-luciferase reporter gene expressions. All of the statistical analyses were performed by SAS 9.1.3 software (SAS Institute, Cary, NC, USA). All statistical tests were two-sided, and the significance level was set at $P < 0.05$.

Results

Characteristics and clinical features of cases and controls

The frequency distributions of selected characteristics of the cases and controls are presented in Table I. They appeared to be well matched on age and sex (P = 0.753 and 0.832, respectively). In addition, there were no significant differences between the cases and controls regarding to BMI and drinking status. However, more smokers, hypertension patients and diabetics were presented in the cases compared with controls (P = 0.035, <0.001 and <0.001, respectively). The majority of patients (84.4%) had the conventional clear cell carcinoma. When stratified according to clinical stage, 62.8%, 19.6%, 7.3% and 10.3% of the patients had stage I, II, III and IV, respectively. The percent of nuclear grade from I to IV was 19.2%, 48.0%, 24.5% and 8.3%, respectively.

Association and stratification analyses between miR-34b/c *rs4938723 polymorphism and RCC risk*

Genotype and allele frequencies distributions of *miR-34b/c* rs4938723 polymorphism are shown in Table II. All perceived genotype frequencies in both controls and cases conformed to Hardy–Weinberg equilibrium (P = 0.116 and 0.838, respectively). The rs4938723C allele frequency was 0.346 in the cases and 0.311 in the controls, and the difference was also significant (P = 0.038). The frequencies of the TT, TC and CC genotypes were 42.5%, 45.6% and 11.8%, respectively, among the cases; and were 46.3%, 45.3% and 8.4%, respectively, among the controls. When the rs4938723TT genotype was used as the reference, we found that the CC genotype was associated with a statistically significant increased risk of RCC (adjusted OR = 1.53, 95% CI = 1.06–2.21). Furthermore, when compared with the combined genotypes (TT and TC), the CC genotype was also associated with a statistically significant increased risk of RCC (adjusted OR = 1.48, 95% CI = 1.05–2.10).

We then evaluated the effect of *miR-34b/c* rs4938723 polymorphism on RCC risk stratified by age, sex, smoking status and drinking status. As shown in Table III, the association between rs4938723 CC genotype and RCC risk appeared stronger in the subgroups of older subjects (OR = 1.80, 95% CI = 1.08–3.01), males (OR = 1.64, 95% CI = 1.08–2.51), ever smokers (OR = 2.07, 95% CI = 1.16–3.69) and ever drinkers (OR = 1.94, 95% CI = 1.01–3.73); but no interaction between rs4938723 polymorphism and these characteristics was observed. We also evaluated the effect of rs4938723 polymorphism on progression of RCC in Table IV. However, no significant difference in clinical stage and grade was observed (data not shown).

Table I. Distribution of selected variables between the renal cell carcinoma cases and control subjects

Variables	Cases (n = 710)		Controls (n = 760)		P[a]
	N	%	*N*	%	
Age (years, mean ± SD)	56.9 ± 11.9		56.8 ± 11.6		0.753
≤57	364	51.3	423	55.7	0.092
>57	346	48.7	337	44.3	
BMI (kg/m^2, mean ± SD)	24.1 ± 2.8		23.8 ± 3.2		0.078
Sex					
Male	454	63.9	490	64.5	0.832
Female	256	36.1	270	35.5	
Smoking status					
Never	444	62.5	515	67.8	**0.035**
Ever	266	37.5	245	32.2	
Drinking status					
Never	508	71.6	571	75.1	0.120
Ever	202	28.5	189	24.9	
Hypertension					
No	444	62.5	444	73.0	**<0.001**
Yes	266	37.5	205	27.0	
Diabetes					
No	611	86.1	716	94.2	**<0.001**
Yes	99	13.9	44	5.8	
Clinical stage					
I	446	62.8			
II	139	19.6			
III	52	7.3			
IV	73	10.3			
Grade					
I	136	19.2			
II	341	48.0			
III	174	24.5			
IV	59	8.3			
Histology					
Clear cell	599	84.4			
Papillary	22	3.1			
Chromophobe	42	5.9			
Unclassified	47	6.6			

Values in bold highlighted the statistical significance.
[a]Student's *t*-test for age and BMI distributions between cases and controls; two-sided χ^2 test for other selected variables between cases and controls.

Table II. Genotype and allele frequencies of *miR-34b/c* rs4938723 among the cases and controls and the associations with risk of renal cell carcinoma

Genotypes	Cases, *n* (%)	Controls, *n* (%)	Crude P[a]	Adjusted P[b]	OR (95% CI)[b]
rs4938723					
TT	302 (42.5)	352 (46.3)			1.00 (reference)
TC	324 (45.6)	344 (45.3)	0.397	0.482	1.08 (0.87–1.35)
CC	84 (11.8)	64 (8.4)	**0.020**	**0.023**	**1.53 (1.06–2.21)**
TT+TC	626 (88.2)	696 (91.6)			1.00 (reference)
CC	84 (11.8)	64 (8.4)	**0.030**	**0.027**	**1.48 (1.05–2.10)**
C allele	34.6	31.1	**0.038**		**1.12 (1.01–1.24)**
P trend					**0.035**

Values in bold highlighted the statistical significance.
[a]Two-sided χ^2 test or Fisher's exact test for either genotype distributions or allele frequencies between the cases and controls.
[b]Allele-specific OR was not adjusted; genotype-specific ORs were adjusted for age, sex, BMI, smoking, drinking status, diabetes and hypertension in logistic regression model.

Effect of miR-34b/c *rs4938723 polymorphism on the expression levels of mature miR-34b/c*

In our study, we collected 27 normal tumour-adjacent tissue samples adjacent to analyse the association between the expression of miR-34b/c and rs4938723 polymorphism. According to the genotyping results, the frequency distributions of the TT, TC and CC were 8, 14 and 5, respectively. As shown in Figure 1, individuals carrying the T allele (TT+TC) had higher expression levels of miR-34b-3p and miR-34c-5p ($P < 0.01$ for TT+TC vs. CC in both miR-34b-3p and miR-34c-5p, respectively). These results suggest that the variant of rs4938723 located in the promoter may regulate the transcriptional activity and then influence the expression levels of miR-34b/c.

Functional results of miR-34b/c *rs4938723 polymorphism in luciferase reporter gene assays*

To explore further whether the rs4938723 polymorphism has an effect on the activity of *pri-miR-34b/c* promoter, reporter gene plasmids were constructed containing either rs4938723 T or rs4938723 C allele and both of them were transiently transfected into 293-T cells. As shown in Figure 2, the vector with the rs4938723 T allele had an increased luciferase activity

Table III. Stratification analyses between *miR-34b/c* rs4938723 genotypes and risk of renal cell carcinoma

Variables	rs4938723 genotypes				P^a	Adjusted OR (95% CI)[b]	$P_{interaction}$
	TT+TC (*n*, %)		CC (*n*, %)				
	Cases	Controls	Cases	Controls			
Age							
≤57	325 (89.3)	386 (91.3)	39 (10.7)	37 (8.8)	0.352	1.27 (0.78–2.05)	0.370
>57	301 (87.0)	310 (92.0)	45 (13.0)	27 (8.0)	**0.034**	**1.80 (1.08–3.01)**	
Sex							
Male	394 (86.8)	447 (91.2)	60 (13.2)	43 (8.8)	**0.029**	**1.64 (1.08–2.51)**	0.499
Female	232 (90.6)	249 (92.2)	24 (9.4)	21 (7.8)	0.513	1.43 (0.67–2.33)	
Smoking status							
Never	400 (90.1)	472 (91.7)	44 (9.9)	43 (8.4)	0.402	1.26 (0.81–1.96)	0.219
Ever	226 (85.0)	224 (91.4)	40 (15.0)	21 (8.6)	**0.024**	**2.07 (1.16–3.69)**	
Drinking status							
Never	454 (89.4)	525 (91.9)	54 (10.6)	46 (8.1)	0.146	1.37 (0.90–2.08)	0.601
Ever	172 (85.2)	171 (90.5)	30 (14.9)	18 (9.5)	0.109	1.94 (1.01–3.73)	

Values in bold highlighted the statistical significance.
[a]Two-sided χ^2 test or Fisher's exact test for the distributions of genotypes.
[b]Adjusted for age, sex, BMI, pack-years of smoking, drinking status, hypertension and diabetes in logistic regression model.

Table IV. Association between *miR-34b/c* rs4938723 polymorphism and progression of renal cell carcinoma

Variables	rs4938723 Genotypes		P^a	Adjusted OR (95% CI)[b]
	TT+ TC (*n*, %)	CC (*n*, %)		
Clinical stage				
Localised (I + II)	388 (87.0)	58 (173.0)	0.208	1.00 (reference)
Advanced (III + IV)	238 (90.1)	26 (9.9)		0.71 (0.43–1.16)
Grade				
Well differentiated (I + II)	413 (86.6)	64 (9.0)	0.158	1.00 (reference)
Moderately differentiated (III)	160 (92.0)	14 (8.1)		0.56 (0.31–1.03)
Poorly differentiated (IV)	53 (89.8)	6 (10.2)		0.65 (0.24–1.60)

[a]Two-sided χ^2 test or Fisher's exact test for the distributions of genotypes.
[b]Adjusted for age, sex, BMI, pack-years of smoking, drinking status, hypertension and diabetes in logistic regression model.

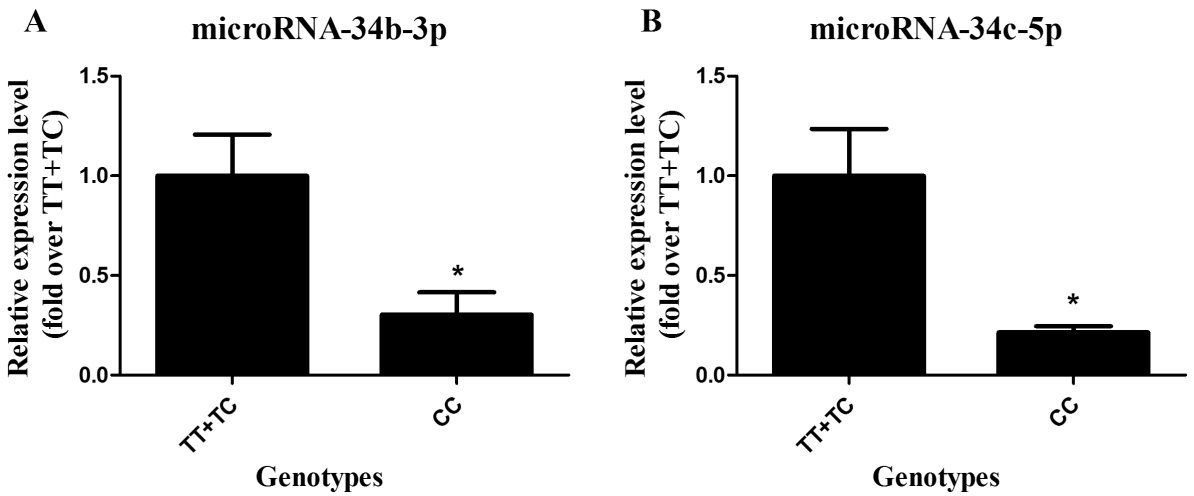

Fig. 1. Expression of miR-34b/c in different genotypes of adjacent normal renal tissues. (**A**) Association between microRNA-34b-3p expression and rs4938723 genotypes. (**B**) Association between microRNA-34c-5p expression and rs4938723 genotypes. The expression levels of both miR-34b and miR-34c were significantly higher in rs4938723 TT+TC group than CC group. The data are expressed as mean ± standard deviation. *$P < 0.01$ vs. CC for both miR-34b and miR-34c.

relative to those with the rs4938723 C allele ($P < 0.05$). The results suggest that the rs4938723 C allele may decrease the expression of miR-34b/c by altering the promoter activities.

Discussion

To our knowledge, this is the first study to evaluate the association between *miR-34b/c* rs4938723 polymorphism and RCC risk in a Chinese population. We found that the CC genotype was associated with a statistically significant increased risk of RCC in the Chinese population, and the risk was more evident in subgroups of older subjects, males, smokers and drinkers. However, no significant difference was found in different clinical stages and grades.

Accumulating studies have shown that SNPs in the promoter of genes can affect the binding efficiency or disrupt the binding

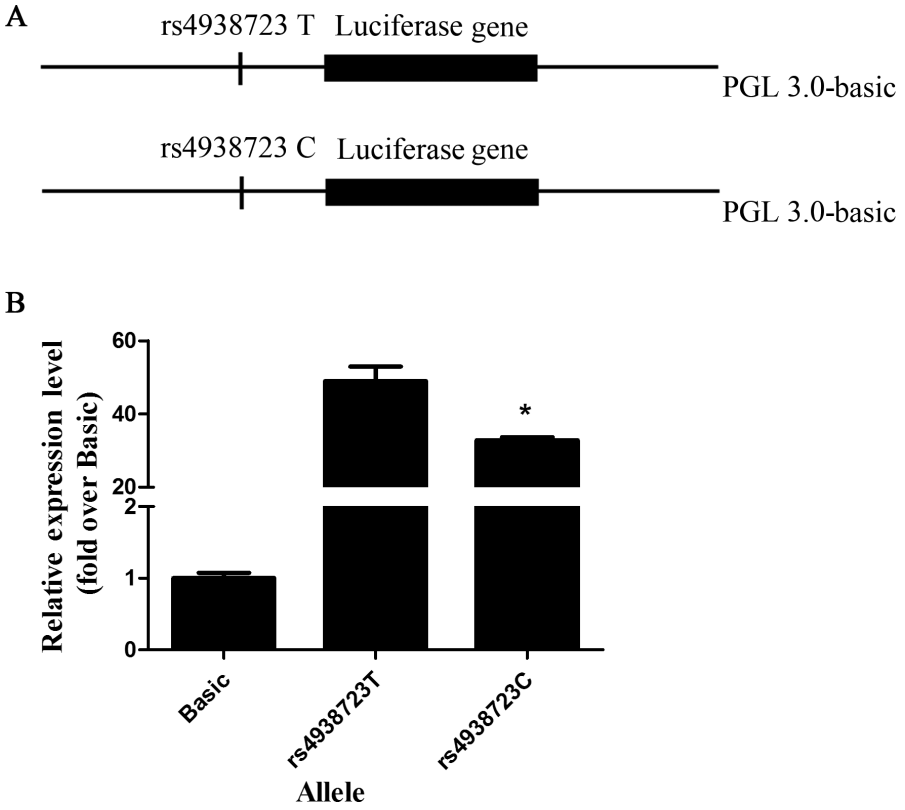

Fig. 2. Effect of rs4938723 polymorphism on the pri-miR-34b/c promoter activity. (**A**) Schematic representation of reporter plasmids carrying the rs4938723 T or C allele, which was inserted into the pGL3 basic plasmid upstream of the luciferase reporter gene. (**B**) Both two plasmids and pGL3 basic vector (as negative control) were transiently transfected into 293-T cells. Renilla luciferase plasmids were co-transfected as internal control to normalise the firefly luciferase activity. The values are expressed as mean ± standard deviation. Columns, mean from three independent experiments; Bars, standard deviation. *$P < 0.05$ for rs4938723 T vs. rs4938723 C allele in 293-T cell line.

of transcriptional factors or other transcriptional elements, which subsequently change the transcription activities of the promoter and the expression level of targeted genes (20–22). Because the rs4938723 is located within the CpG island of *pri-miR-34b/c* and may create a predicted GATA-binding site, it may affect the expression of *miR34b/c* by both genetic and epigenetic mechanisms. Frequent concomitant inactivation of *miR-34b/c* induced by CpG methylation was reported in several cancers previously, including RCC (13). Additionally, in our study, we found that the miR-34b/c expression levels decreased in subjects with rs4938723 C allele. Luciferase reporter gene assay confirmed that *pri-miR-34b/c* promoter rs4938723 T to C substitution could significantly decrease the transcriptional activity of *pri-miR-34b/c*. Taking these findings together, it is biologically plausible that the promoter polymorphism rs4938723 T>C in *miR-34b/c* might affect individuals' susceptibility to RCC by creating the predicted GATA-binding site or the CpG methylation.

To date, there have been three studies investigating the association between the *miR-34b/c* polymorphisms and cancer risk. Son *et al.* (17) indicated that the T allele in *miR-34b/c* T>C (rs4938723) was associated with the decreased risk of hepatocellular cell carcinoma in the Korean population. Gao *et al.* (18) suggested that the rs4938723 in the promoter region of pri-miR-34b/c played a protective role in the development of colorectal cancer. In support of our results, Xu *et al.* (16) previously suggested that the variant genotype of rs4938723 (CC genotype) was associated with increased risk for hepatocellular carcinoma. Their study was also conducted in a Chinese population, and the frequency of rs4938723 C allele in their controls was 30.7%, similar to that in our controls (31.1%). In the gene–environment interaction analysis, they found a statistically significant interaction between *miR-34b/c* rs4938723 and drinking status on hepatocellular carcinoma risk. In our study, although we observed that ever drinkers carrying the variant CC genotype had a 1.94-fold increased risk of RCC, no interaction effect between this SNP and drinking status was found ($P_{interaction} = 0.601$). Alcohol consumption has been considered as a major risk factor for HCC but not for RCC (23); therefore, the different role of alcohol in the aetiology of these two diseases may contribute to the disparity. In addition, we also observed that older male subjects carrying the variant genotype of the *miR-34b/c* rs4938723 was at significantly increased risk for RCC, suggesting these subgroups may be inherently more susceptible to certain carcinogens. Cigarette smoking is the most consistently established causal risk factor for RCC and accounts for ~20% of RCC (5). Interestingly, our results indicated that smokers with the risk genotype of *miR-34b/c* rs4938723 had a 2.07-fold increased risk of RCC, though no interaction between smoking status and rs4938723 was observed ($P_{interaction} = 0.219$).

In conclusion, we found that rs4938723 C allele in the promoter of *miR-34b/c* was associated with increased risk of RCC in our population by decreasing the transcriptional activity of *pri-miR-34b/c*. These initial findings need to be independently validated by other large independent population-based studies.

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