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Association between a functional variant in microRNA-27a and susceptibility to colorectal cancer in a Chinese Han population

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ABSTRACT. Polymorphisms in pri-, pre-, and mature-microRNAs (miRNAs) have been proposed to be associated with various human cancers. Common single nucleotide polymorphisms (SNPs) in miRNA genes can influence the maturation of miRNAs or miRNA-mediated transcriptional regulation. Through genotyping the rs895819 SNP in 254 colorectal cancer (CRC) patients and 238 healthy controls by polymerase chain reaction-restricted fragment length polymorphism, a case-control study was performed to investigate a possible association between a common A/G polymorphism (rs895819) within *hsa-mir-27a* and susceptibility to CRC in a Chinese Han population. In addition, after examining miR-27a expression levels in CRC tissues (N = 57) obtained from these CRC patients, we found that subjects with variant genotypes (AG+GG) had a significantly increased risk of developing

CRC compared to AA carriers (odds ratio (OR) = 1.619, 95% confidence interval (CI) = 1.129-2.322). The elevated risk was especially evident in older (age ≥ 60 years) and male subjects. Further functional analyses indicated that the relative expression of miR-27a was significantly greater in tumor tissues from GG patients or patients carrying at least one G allele than in those from AA patients. In conclusion, we provide the first evidence that an miR-27a polymorphism contributes to CRC susceptibility in the Chinese Han population by modulating mature miR-27a expression.

Key words: Colorectal cancer; MicroRNA-27a; Polymorphisms

INTRODUCTION

Colorectal cancer (CRC) is one of the most common cancers of the gastrointestinal tract worldwide (Jemal et al., 2009). The incidence and mortality of CRC in China have increased rapidly over the past several decades (Yang et al., 2003). Studies have demonstrated that both genetic and environmental factors contribute to CRC development (Lichtenstein et al., 2000). According to recent studies, several other factors, such as alcohol consumption and tobacco use (Hoshiyama et al., 1993; Tsong et al., 2007), dietary and lifestyle factors (Huxley et al., 2009), and inflammatory bowel disease (Eaden et al., 2001; von Roon et al., 2007), have also been associated with CRC risk. Although many people are exposed to similar risk factors, only a few of the exposed individuals develop CRC, indicating that genetic variation also partially influences individual susceptibility to colorectal tumorigenesis.

Accumulating evidence has shown that microRNAs (miRNAs) play important roles in various biological processes, including cell proliferation, apoptosis, tumorigenesis, and multidrug resistance of cancers (Zhang et al., 2010). As a class of 22-nucleotide noncoding RNAs, miRNAs are thought to function as negative regulators of gene expression. Various single nucleotide polymorphisms (SNPs) exist in miRNA genes that can lead to variations in the quantity of miRNAs, resulting in diverse functional consequences that include cancer development (Horikawa et al., 2008). For example, a study by Zhang et al. (2010) provided evidence that an miR-196a2 polymorphism might contribute to CRC susceptibility in Chinese populations through modulating mature miR-196a expression. This functional polymorphism was also shown to be associated with susceptibility to lung (Tian et al., 2009), breast (Hu et al., 2009), and gastric cancer (Peng et al., 2010).

Here, we aimed to determine whether the polymorphism rs895819, a functional variant in microRNA-27a (miR-27a), was associated with an increased risk of developing CRC in a Chinese Han population.

MATERIAL AND METHODS

Subjects

A hospital-based case-control study was undertaken. We enrolled 254 unrelated

CRC patients in this study between April 2009 and September 2011. The diagnosis of CRC was confirmed by histopathological examination. Patients with recurring CRC and a history of cancers or other serious diseases were excluded. The control group included 238 unrelated Chinese Han individuals from the same geographical area. These healthy controls were randomly selected from a pool of healthy volunteers who visited the general health check-up center during the same time period. Control subjects with a family history of cancer or serious diseases, such as gastritis, ulcerative colitis, Crohn's colitis, hypertension, or diabetes, were excluded.

Genotyping

DNA was isolated from peripheral blood using the Blood Genome DNA Extraction Kit (TaKaRa; Dalian, China). Genotyping was performed by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The 182-bp DNA fragment containing the polymorphic site was amplified using two primers: 5'-GAACTTAGCCACTGTGAACACCACTTGG-3' (forward) and 5'-TTGCTTCCTGTCAC AAATCACATTG-3' (reverse). The reaction mixtures were denatured at 95°C for 5 min, followed by 35 cycles of 95°C for 25 s, 60°C for 30 s, and 72°C for 30 s, with a final elongation at 72°C for 10 min. The PCR products were digested with *Dra*III (New England Bio-Labs; Waltham, MA, USA) at 37°C overnight followed by 3% agarose gel electrophoresis. The genotypes were assessed as follows: a single 182-bp fragment represented the GG genotype; two fragments of 155 and 27 bp represented the AA genotype; and three fragments of 182, 155, and 27 bp represented the AG genotype.

Stem-loop reverse transcription (RT)-PCR for miR-27a

For miR-27a expression, we obtained 57 tumor tissue samples with histological evidence of primary colorectal carcinoma from the patients enrolled in this study. All tissues were snap-frozen in liquid nitrogen after surgical resection and stored at -80°C until RNA extraction. Total RNA was extracted with Trizol reagent (Invitrogen; Carlsbad, CA, USA). As an internal control, the universally expressed U6 small nuclear RNA was used for miR-27a template normalization. Details of primers and PCR conditions were described previously (Li et al., 2010). All reactions were performed in triplicate. The expression of miR-27a was analyzed using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

All analyses were performed using the SPSS statistical software package version 12.0 (SPSS Inc.; Chicago, IL, USA). Numeric values were analyzed with the Student *t*-test. Hardy-Weinberg equilibrium among the populations studied was evaluated with the χ^2 test. Differences in characteristics between CRC cases and controls were assessed with the Fisher exact test, as were disparities in genotype and allele frequencies. We calculated the odds ratios (ORs) and 95% confidence intervals (95%CIs) to estimate the risk of developing CRC. Instances where $P < 0.05$ were considered to represent statistically significant differences.

RESULTS

Baseline characteristics

There were a total of 254 CRC patients and 238 healthy controls in this case-control study. Baseline characteristics of our patient cohort are summarized in Table 1. There were no statistically significant differences between the cases and controls in terms of gender, age, smoking status, and alcohol consumption, suggesting that our frequency of matching of the demographic characteristics in our patient groups was satisfactory.

Table 1. Characteristics of the subjects in both groups.

	Patients (N, %)		Controls (N, %)		P value
Age (years)					
≥60	167	(65.7%)	162	(68.1%)	0.632
<60	87	(34.3%)	76	(31.9%)	
Gender					
Male	182	(71.7%)	177	(74.4%)	0.543
Female	72	(28.3%)	61	(25.6%)	
Smoking status					
Ever	143	(56.3%)	135	(56.7%)	0.928
Never	111	(43.7%)	103	(43.3%)	
Alcohol drinking					
Ever	112	(44.1%)	107	(45.0%)	0.856
Never	142	(55.9%)	131	(55.0%)	

SNP rs895819 and the risk of developing CRC

Genotype distributions of SNP rs895819 in patients and controls were in agreement with Hardy-Weinberg equilibrium (for controls $\chi^2 = 2.894$, $P = 0.089$; for patients $\chi^2 = 1.792$, $P = 0.181$). Distribution of the rs895819 genotype and associated risks of developing CRC are presented in Table 2. The frequency of the rs895819 GG genotype was higher in the CRC group than in the control group (19.3 *vs* 13.0%). The frequency of the G allele was also significantly higher in the CRC patient group (41.5%) than in the control group (32.6%). These data implied that subjects with the GG genotype or G allele-containing genotypes (GG and AG) might have a higher risk of developing CRC. These results indicated that the miR-27a polymorphism was associated with an increased susceptibility to CRC in a Chinese Han population (Table 2).

Table 2. Distributions of the rs895819 genotype.

Genotype	Cases (N, %)	Controls (N, %)	P value; OR (95%CI)
rs895819 A/G	254	238	
AA	92 (36.2%)	114 (47.9%)	
AG	113 (44.5%)	93 (39.1%)	0.049; 1.506 (1.021-2.220)
GG	49 (19.3%)	31 (13.0%)	0.013; 1.959 (1.156-3.318)
AG+GG	162 (63.8%)	124 (52.1%)	0.010; 1.619 (1.129-2.322)
A allele	297 (58.5%)	321 (67.4%)	
G allele	211 (41.5%)	155 (32.6%)	0.004; 1.471 (1.134-1.909)

OR = odds ratio; CI = confidence interval.

Statistical analyses

Table 3 shows the results of stratified analyses by age, gender, smoking status, and alcohol consumption for the SNP. A statistically significant association between increased CRC risk and rs895819 variant genotypes was observed in older (age ≥ 60 years) subjects (OR = 1.700, 95%CI = 1.092-2.645) and male subjects (OR = 1.788, 95%CI = 1.169-2.736). No significant association was observed between smoking status or alcohol consumption with the risk of developing CRC.

Table 3. Stratified analyses for variant rs895819 genotypes.

	(AG+GG)/AA		OR (95%CI)	P value
	Cases	Controls		
Age (years)				
≥60	108/59	84/78	1.700 (1.092-2.645)	0.019
<60	54/33	40/36	1.473 (0.789-2.751)	0.267
Gender				
Male	120/62	92/85	1.788 (1.169-2.736)	0.007
Female	42/30	32/29	1.269 (0.638-2.522)	0.600
Smoking status				
Ever	82/61	62/73	1.583 (0.986-2.542)	0.072
Never	80/31	62/41	1.707 (0.963-3.025)	0.082
Alcohol drinking				
Ever	62/50	46/61	1.644 (0.964-2.805)	0.079
Never	100/42	78/53	1.618 (0.980-2.672)	0.075

OR = odds ratio; CI = confidence interval.

Effect of the SNP rs895819 on miR-27a expression

To determine whether the SNP rs895819 identified in *hsa-mir-27a* could affect mature miR-27a expression, we measured the expression level of mature miR-27a in 57 tumor tissues. Among the respective 57 patients, 22 carried the rs895819 AA genotype, 28 carried the AG genotype, and 7 carried the GG genotype. Using RT-PCR, we found that the expression of miR-27a was significantly greater in tumor tissues from GG patients or patients carrying at least one G allele than in those from AA patients ($P < 0.05$) (Figures 1 and 2).

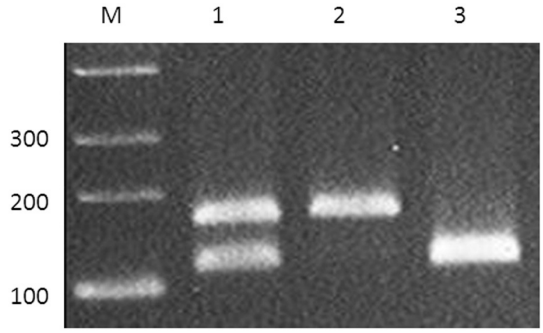

Figure 1. PCR-RFLP analysis of single-nucleotide polymorphism rs895819 in *hsa-mir-27a*. *Lane M* = DNA marker; *lane 1* = AG heterozygous; *lane 2* = GG homozygous; *lane 3* = AA homozygous.

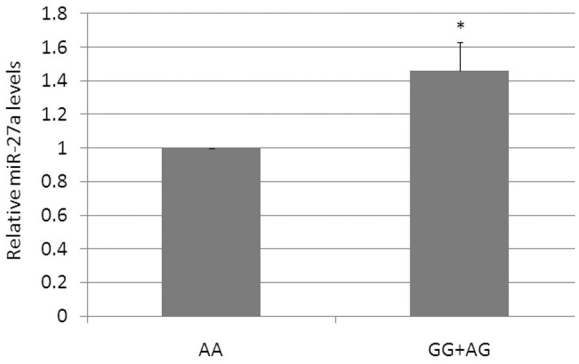

Figure 2. Relative expression levels of miR-27a in variant rs895819 genotypes.*P < 0.05.

DISCUSSION

To our knowledge, this is the first study to evaluate the relationship between the rs895819 SNP in *hsa-mir-27a* and CRC risk in a Chinese Han population. We observed that the G allele was significantly associated with an increased risk of developing CRC. Although it is not possible at this time to further elucidate the mechanism of cancer predisposition for patients who harbor this G allele, our findings indicate that the polymorphism in *hsa-mir-27a* could play a role in CRC development and might contribute to a pathogenic mechanism.

Polymorphisms in the miRNA coding sequence (miR-polymorphisms) or SNPs in the miRNA binding sites are a novel class of functional polymorphisms present in the human genome. These polymorphisms can influence miRNA expression or modulate target gene expression (Bertino et al., 2007; Mishra et al., 2007, 2008). In a relatively short time (less than 2 years), several research groups from around the globe have established the importance of miR-polymorphisms and suggested a strong association of miR-polymorphisms with disease progression and drug responses (Mishra and Bertino, 2009).

Increasing evidence has shown that miRNA can influence susceptibility to certain diseases, including cancers, by regulating gene expression levels. The miR-27a gene, located on chromosome 19, has been shown to be involved in the development of gastric adenocarcinoma by targeting prohibitin (Liu et al., 2009). In addition, it was found to suppress the expression of *zinc finger and BTB domain containing 10* (*ZBTB10*) mRNA by interfering with Sp1 activation (Tillotson, 1999; Scott et al., 2006). Studies have demonstrated that expression and activation of the transcription factor Sp1 can contribute to cancer cell survival, growth, and angiogenesis (Zhu et al., 2006). Rs895819 was one A/G polymorphism found in *hsa-mir-27a*, and it was located at position 40 relative to the first nucleotide. Sun et al. (2010) found that a common polymorphism (rs895819) acted as an important factor in gastric cancer susceptibility by modulating miR-27a and *ZBTB10* levels. However, the relationship between rs895819 and susceptibility to CRC remains unclear. In our case-control study, we found that subjects with the variant genotype AG+GG showed a significantly increased risk of developing CRC compared to AA carriers (OR = 1.619, 95%CI = 1.129-2.322; P = 0.010). The elevated risk was especially evident in older (age ≥ 60 years) and male subjects. Further functional analyses

indicated that the relative miR-27a expression level was significantly higher in tumor tissues from GG patients or patients carrying at least one G allele than in those from AA patients ($P < 0.05$). Our results showed that rs895819 could influence susceptibility to CRC by modulating miR-27a levels.

There were several limitations to our study. First, our sample size was relatively small, particularly for miRNA expression analyses. Second, although only mature miR-27a levels were examined, the miR-27a SNP might also influence the processing of pri- and/or pre-miRNA, and, thus, the expression levels of pri- and pre-miRNA still need to be determined. Finally, dietary habits play an important role in the development of CRC, and we did not obtain any information on dietary habits from the subjects in our study. Therefore, further studies with a larger sample size and more parameters should be conducted. However, our observations provide useful information and may guide future studies in this area.

In summary, our study provides initial evidence that genetic variants in *hsa-mir-27a* confer an increased risk of developing CRC in a Chinese Han population, possibly by increasing the levels of mature miR-27a. We believe that our findings provide novel insights into the molecular mechanisms of colorectal tumorigenesis.

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