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Original Article

Association of hsa-miR-423 rs6505162 polymorphism with risk of nasopharyngeal carcinoma in Southern Chinese population

Binbin Li[1,2*], Huahui Li[2*], Weiping Ye[3], Xiaoyan Huang[3], Yang Han[3], Jiaxu Wang[3], Shaopeng Du[3], Guoliang Huang[2], Zhiwei He[1,2]

[1]Department of Pathophysiology, [2]Key Laboratory for Epigenetics of Dongguan City, [3]The Second Clinical Medicine Faculty, Guangdong Medical University, Dongguan, Guangdong, China. [*]Equal contributors.

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Abstract: Objectives: The single-nucleotide polymorphisms (SNPs) occurring in microRNAs (miRNAs) are novel sources of genetic variation which may contribute to cancer susceptibility and prognosis. This study aimed to estimate the association between hsa-miR-423 polymorphism and risk of nasopharyngeal carcinoma (NPC) in Southern Chinese population. Methods: In a case-control study, a total of 406 patients with NPC and 418 healthy controls were genotyped using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. The genetic associations with the risk of occurrence and progression of NPC were estimated by logistic regression analysis. Results: Compared with CC/CA genotype, miR-423 rs6505162 AA genotype exhibited significant association with an increased risk of NPC (AA *vs.* CC: adjusted OR=2.056, 95% CI 1.126-3.751, P=0.019; AA *vs.* CA: adjusted OR=1.970, 95% CI 1.067-3.637, P=0.030; AA *vs.* CC/CA: adjusted OR=2.027, 95% CI 1.125-3.652, P=0.019, respectively). No significant association was observed between the genotypes and clinical characteristics of NPC patients. Conclusions: The functional polymorphism rs6505162 in miR-423 might alter individual susceptibility to NPC in Southern Chinese population. CC genotype or C allele might be a protective factor for NPC.

Keywords: MiR-423, nasopharyngeal carcinoma, single-nucleotide polymorphism

Introduction

Nasopharyngeal carcinoma (NPC) is an epithelial malignancy with an unusual difference in ethnic and geographical distributions. Although it is rare in most parts of the world, the prevalence rate of NPC is remarkably high in Southern China and other countries of Southeast Asia, with the average incidence of 15 to 50 per 100,000 [1]. The etiology of NPC is thought to be tied with a complex interaction of genetic susceptibility and environmental factors, including Epstein-Barr virus (EBV) infection, exposure to chemical carcinogens and dietary habits [2]. Familial aggregation is a notable epidemiological feature of NPC [3].

MicroRNAs (miRNAs) are small non-coding RNAs involved in post-transcriptional regulation of gene expression by binding to 3'-untranslated regions (UTRs) of their target mRNAs. MiRNAs play important roles in various biological processes such as proliferation, differentiation, development, and metabolism through modulating approximately one-third of human genes [4]. Dysregulation of miRNAs was closely correlated with the initiation and progression of various types of cancers, including NPC [5, 6]. The single-nucleotide polymorphisms (SNPs) within miRNA or pre-miRNA sequences have been demonstrated to affect the expression or biological functions of mature miRNAs, consequently contributing to cancer susceptibility and prognosis [7]. Several studies have shown that some SNPs of miRNAs are associated with NPC risk and outcome. The SNPs in miR-146a rs2910164 and miR-196a2 rs11614913 may contribute to the risk and progression of NPC in Chinese population [8, 9]. MiR-608 rs4919510 C > G may be a predictive marker to identify radiotherapy-treated NPC patients with a high

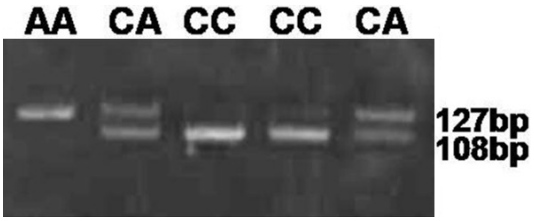

Figure 1. Genotypes of miR-423 rs6505162 identified by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. One band (127 bp) for AA homozygote genotype, two bands (108 and 19 bp) for CC homozygote genotype, three bands (127, 108 and 19 bp) for CA heterozygote genotype.

risk of recurrence [10]. However, potential roles of these SNPs in NPC tumorigenesis remain to be elucidated.

MiR-423 gene is located on chromosome 17q11.2 and can generate two mature transcripts, named miR-423-3p and miR-423-5p. MiR-423 has been shown to play important roles in development, progression, and prognosis of multiple human cancers [11-13]. A common C/A polymorphism rs6505162 is located within the pre-miR-423, 12 base pairs 5' of miR-423-5p. The association between the rs6505162 SNP in pre-miR-423 and cancer risk has been evaluated in a range of cancers from diverse populations, including breast cancer, esophageal cancer, hepatocellular cancer, ovarian cancer and bladder cancer, but the findings are still contradictory [14-18]. The roles of miR-423 genetic variant in NPC have never been specifically investigated thus far. In this study, using the case-control analysis, the frequency of SNP rs6505162 in pre-miR-423 was analyzed, and the associations with the risk of occurrence and progression of NPC were evaluated in Southern Chinese population.

Materials and methods

Study population

This case-control study included 406 patients with NPC and 418 healthy controls at Affiliated Hospital of Guangdong Medical University (Zhanjiang, China) from January 2013 to October 2015. The patients were diagnosed by histopathology evidence and received no treatment before the blood drawing. Tumor size, nodal status and distant metastasis were clinically determined by computed tomography (CT) scan or magnetic resonance imaging (MRI). Clinical stages were assessed with the criteria of Union for International Cancer Control (UICC 2010). Controls were genetically unrelated cancer-free individuals who underwent routine medical examination in the same hospital. All subjects were natives of Guangdong Province. This study protocol was approved by Ethics Committees of Guangdong Medical University (Zhanjiang, China), and written informed consent was obtained from all the participants involved.

Isolation of DNA and genotyping

Genomic DNA was extracted from peripheral blood samples on the basis of standard procedures (Tiangen Biotech, Beijing, China). Genotyping was performed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. The PCR mixture consisted of 1× GoTaq Master Mix (Promega Corporation, USA), 2.5 pmol of each of the forward and reverse primers and 50 ng of template DNA in a 15 μl reaction volume. The primers of pre-miR-423 were as follows: forward, 5'-CCCCTCAGTCTTGCTTCGTA-3' and reverse, 5'-ACTTGAGCTTCTGCCAAGGA-3'. The PCR products of miR-423 were digested with Csp6 I (Thermo, USA) at 37°C for more than 6 h or overnight. After that, the cleaved products were separated on polyacrylamide gel electrophoresis and identified by ethidium bromide staining. For the miR-423 rs6505162 polymorphism, CC homozygote genotype was cleaved to be two bands (108 and 19 bp), CA heterozygote genotype to be three bands (127, 108 and 19 bp), AA homozygote genotype to be one band (127 bp) (**Figure 1**). The sequences of PCR products were confirmed by DNA sequencing in about 5% of the samples (**Figure 2**).

Statistical analysis

The diverse characteristics between patients and controls were analyzed by t-test or chi-square test. The Hardy-Weinberg equilibrium was utilized to compare the observed genotype frequencies with the expected ones in the control group. The different distribution of the gen-

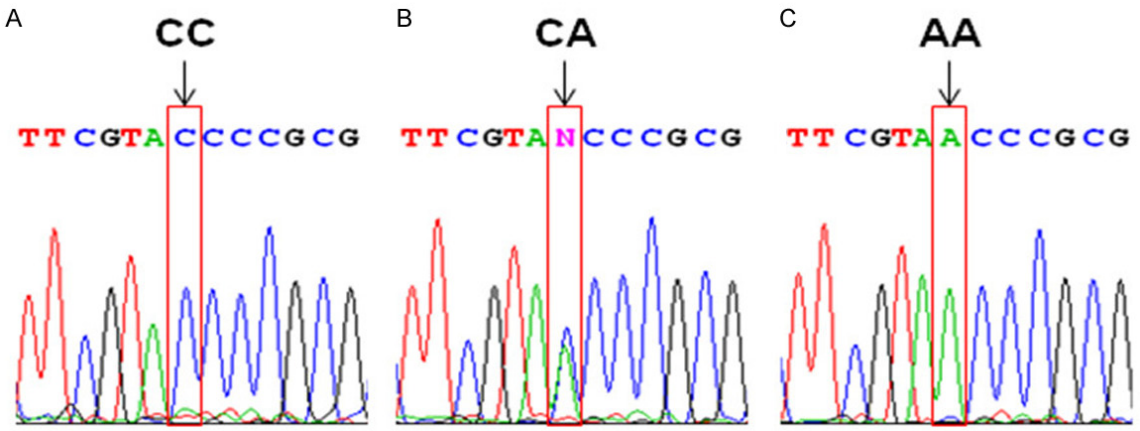

Figure 2. DNA sequences of miR-423 rs6505162 genotypes harbouring CC homozygotes (A), CA heterozygotes (B) and AA homozygotes (C).

Table 1. Characteristics of the participants in the case-control study

Characteristics	Patients	Controls	t or χ^2-value	*P* value
Mean age (mean ± SD)	48.02±11.57	48.51±10.33	0.645	0.519
Age				
< 45	163 (40.1)	154 (36.8)	0.951	0.330
≥ 45	243 (59.9)	264 (63.2)		
Gender				
Male	291 (71.7)	291 (69.6)	0.420	0.541
Female	115 (28.3)	127 (30.4)		
T stage*				
T1 + T2	122 (37.0)			
T3 + T4	208 (63.0)			
N stage*				
N0	24 (7.3)			
N1-N2-N3	306 (92.7)			
Metastasis*				
No	300 (90.9)			
Yes	30 (9.1)			
Clinical stage*				
I + II	27 (8.2)			
III + IV	303 (91.8)			

Abbreviations: SD, standard deviation. *The sum of patients with certain characteristics does not equal the total number of patients due to unavailable data.

otype frequencies between patients and controls was evaluated by chi-square test. The associations between the rs6505162 genotypes and the risk or progression of NPC were estimated by calculating the odds ratios (ORs) and 95% confidence intervals (CIs), using the multivariate logistic regression analysis adjusted by age and gender. All statistical analyses were performed with SPSS 19.0 software and P<0.05 was considered to indicate statistical significance.

Results

Characteristics of the population in the case-control study

A total of 406 patients with NPC and 418 healthy controls from Guangdong province in Southern China were recruited in this study. Main characteristics of the study subjects were present in **Table 1**. There were no statistically significant differences between NPC patients and controls in terms of the distributions of age (P=0.207) and gender (P=0.517). A vast majority of patients were classified as poorly differentiated squamous cell carcinoma. More than 90% of patients were in the late clinical stage according to TNM stage (**Table 1**).

Distribution of genotype and the association with risk of NPC

The distribution of genotype and allele frequencies of miR-423 rs6505162 in patients and

Table 2. Genotype and allele distribution of miR-423 rs6505162 in NPC patients and controls

Polymorphism	Patient	Control	*P* value[a]	Crude OR (95% CI)	β value	*P* value	Adjusted OR (95% CI)[b]	β value[b]	*P* value[b]
rs6505162 C > A									
Genotype									
CC	216 (53.2)	238 (56.9)		1.000			1.000		
CA	156 (38.4)	162 (38.8)		1.061 (0.796-1.413)	0.059	0.686	1.052 (0.788-1.404)	0.050	0.732
AA	34 (8.4)	18 (4.3)		2.081 (1.142-3.793)	0.733	0.017	2.056 (1.126-3.751)	0.721	0.019
CA+AA	190 (46.8)	180 (43.1)		1.163 (0.884-1.531)	0.151	0.281	1.149 (0.871-1.514)	0.138	0.326
AA vs. CA vs. CC			0.052						
CA	156 (38.4)	162 (38.8)		1.000			1.000		
AA	34 (8.4)	18 (4.3)		1.962 (1.064-3.618)	0.674	0.031	1.970 (1.067-3.637)	0.678	0.030
CC+CA	372 (91.6)	400 (95.7)		1.000			1.000		
AA	34 (8.4)	18 (4.3)		2.031 (1.128-3.659)	0.709	0.018	2.027 (1.125-3.652)	0.706	0.019
Allele									
C	588 (72.4)	638 (76.3)		1.000					
A	224 (27.6)	198 (23.7)		1.228 (0.984-1.532)	0.205	0.070			

Abbreviations: CI, confident interval; OR, odd ratio. [a]*P* value was calculated from two-sided chi-square test. [b]Data were calculated by logistic regression with adjustment for age and gender.

Table 3. Associations between miR-423 rs6505162 genotypes and clinicopathological characteristics of NPC patients

Parameters	rs6505162 C > A			*P* value[a]	AA vs. CC		CA vs. CC	
	AA	CA	CC		OR (95% CI)	*P* value	OR (95% CI)	*P* value
Age								
< 45	16 (9.8)	66 (40.5)	81 (49.7)	0.447	1.000		1.000	
≥ 45	18 (7.4)	90 (37.0)	135 (55.6)		0.673 (0.325-1.396)	0.287	0.819 (0.538-1.244)	0.349
Gender								
Male	26 (8.9)	118 (40.5)	147 (50.5)	0.225	1.000		1.000	
Female	8 (7.0)	38 (33.0)	69 (60.0)		0.661 (0.284-1.538)	0.337	0.710 (0.448-1.126)	0.145
T stage								
T1 + T2	12 (9.8)	59 (48.4)	51 (41.8)	0.847	1.000		1.000	
T3 + T4	18 (8.7)	107 (51.4)	83 (39.9)		0.907 (0.402-2.044)[b]	0.813[b]	1.076 (0.668-1.734)[b]	0.763[b]
N stage								
N0	2 (8.3)	14 (58.3)	8 (33.3)	0.71	1.000		1.000	
N1-N2-N3	28 (9.1)	152 (49.7)	126 (41.2)		1.376 (0.352-5.371)[b]	0.646[b]	0.630 (0.252-1.576)[b]	0.323[b]
Metastasis								
No	27 (9.0)	149 (49.7)	124 (41.3)	0.696	1.000		1.000	
Yes	3 (10.0)	17 (56.7)	10 (33.3)		0.661 (0.284-1.538)[b]	0.337[b]	1.428 (0.629-3.239)[b]	0.394[b]
Clinical stage								
I + II	4 (14.8)	9 (33.3)	14 (51.9)	0.161	1.000		1.000	
III + IV	26 (8.6)	157 (51.8)	120 (39.6)		0.742 (0.224-2.455)[b]	0.625[b]	1.929 (0.797-4.668)[b]	0.145[b]

[a]*P* value was calculated from two-sided chi-square test. [b]The correlation between genotypes and primary tumor extension, regional lymph node status, metastatic status and clinical stage was estimated by logistic regression with adjustment for age and gender.

controls was shown in **Table 2**. The observed genotype distribution of rs6505162 in the controls was in agreement with Hardy-Weinberg equilibrium (χ^2=2.173, P=0.140). The minor allele frequency (MAF) for rs6505162 in our controls (0.237) was similar to MAF for Chinese population in database (0.200) (http://www.ncbi.nlm.nih.gov/projecs/SNP). Based on age- and gender-adjusted logistic regression analysis, the carriers bearing miR-423 AA homozygote genotype showed significant association with an increased risk in NPC susceptibility compared with the ones bearing CC/CA genotype (AA *vs.* CC: adjusted OR=2.056, 95% CI 1.126-3.751, P=0.019; AA *vs.* CA: adjusted OR=1.970, 95% CI 1.067-3.637, P=0.030; AA *vs.* CC/CA: adjusted OR=2.027, 95% CI 1.125-3.652, P=0.019, respectively, **Table 2**). However, compared with CC genotype, the CA genotype for rs6505162 bore no significant suscep-

tibility to NPC (CA *vs.* CC: adjusted OR=1.052, 95% CI 0.788-1.404, P=0.732, **Table 2**). In addition, a marginal difference was observed in allele frequencies between NPC patients and healthy controls (OR=1.228, 95% CI 0.984-1.532, P=0.070, **Table 2**).

Associations between genotypes and clinicopathological characteristics of NPC

We further assessed the effect of miR-423 rs6505162 SNP on the progression and severity of NPC. The relationship of rs6505162 C > A genotype with clinicopathological parameters, including primary tumor extension, regional lymph node status, metastatic status and clinical stages, was analyzed for NPC patients. As shown in **Table 3**, no statistically significant association was observed between the genotypes and clinicopathological parameters in NPC patients.

Discussion

Extensive studies have demonstrated the importance of miRNAs in cancer development as they could act as either oncogenes or tumor suppressor genes, depending on the functions of their targets [5, 19]. MiR-423 was observed to be overexpressed in multiple cancer types, including breast cancer, colorectal cancer, hepatocellular carcinoma, and head and neck squamous cell carcinomas [20-23]. The miR-423-3p, not miR-423-5p, could promote cell proliferation and enhance G1/S transition of cell cycle by targeting p21Cip1/Waf1 in hepatocellular carcinoma and colorectal cancer [21, 22]. Overexpression of miR-423-3p increased cell proliferation, clonogenicity, cell migration and invasion through modulation of adiponectin receptor 2 (AdipoR2) in laryngeal carcinoma [12]. MiR-423-5p negatively regulated the expression of trefoil factor 1 (TFF1), and thus mediated proliferation/invasion-related processes via a TFF1-dependent manner in gastric cancer cells [11]. The levels of circulating miR-423-5p were found to be significantly up-regulated in colorectal cancer and gastric cancer patients, and were correlated with tumor stage [24, 25]. The findings suggested miR-423 might play an important oncogenic role in the occurrence and progression of some cancers. But miR-423 was reported to be reduced in expression in mesothelioma and psoriasis lesions [26, 27].

The SNPs occurring in miRNAs are novel sources of genetic variation, which may affect many cancer-related biological processes though altering their biogenesis, processing, and binding to target mRNAs in a variety of ways [7]. The rs6505162 SNP is located within the pre-miR-423 only, not being present in either of the mature sequences, which indicated that this SNP does not affect the mature miR-423 binding to its target mRNAs, and thus does not influence the expression of those genes. But it is plausible that the SNPs in pri- miRNAs or pre-miRNAs may impact the processing procedure or expression of mature forms [28, 29]. The rs6505162 SNP was shown to be involved in the endogenous processing of pri-miR-423 to its two mature forms. In breast cancer cells, pre-miR-423 C allele appeared to block the processing efficiency by affecting the recognition of cleavage site by DGCR8, which led to dramatically reduced expression of the mature miR-423 [13]. In addition, the rs6505162 SNP is also placed in the pri-miR-3184 which is the complementary miRNA of miR-423 on chromosome 17q11.2. MiR-3184 has been reported to be differentially expressed in miRNA profiles of melanomas [30]. It is possible that the rs6505-162 SNP may affect the expression or functions of miR-3184, and thus mediate some effects of this SNP. Further studies are still needed to uncover the effects of this SNP on expression and functionality of the two miRNAs.

The relationship between the rs6505162 SNP in pre-miR-423 and cancer risk has been evaluated in diverse populations and in a range of cancers, but the results were contradictory. The study conducted by Smith et al showed that the rs6505162 CC genotype was associated with a reduced risk of breast cancer in Caucasian populations [14]. However, the CC genotype or C allele was also reported to contribute to an increased susceptibility to esophageal cancer in Caucasian populations [15]. Similar observations are also shown in the results of Kontorovich et al and Hu et al, where C allele conferred a significantly increased risk of ovarian cancer and bladder cancer [17, 18]. In addition, the C allele of rs6505162 was found to increase the risk of recurrence in patients with renal cell carcinoma and the mortality of prostate cancer [31, 32]. But in a recent meta-analysis, rs6505162 SNP failed to exhibit any significant association with the risk of

breast cancer among Asians [33]. This SNP was also shown to have no effect on the susceptibility of hepatocellular carcinoma (HCC) in East Chinese population [16], despite its expression was significantly up-regulated in HCC tissues [22]. These conflicting results suggested that miR-423 rs6505162 SNP may have varying effects on cancer risk depending on different types of cancers and genetic backgrounds from different ethnicities.

In the present study, we investigated the genetic associations between miR-423 rs6505162 SNP with the risk of occurrence and progression of NPC in Southern Chinese population. We found that the individuals with rs6505162 AA homozygote genotype had a significantly increased susceptibility to NPC compared with the ones bearing CC/CA genotype. The allele A was also shown to be marginally associated with an increased risk of NPC. The results indicated that rs6505162 CC genotype or C allele might serve as a protective role for initiation of NPC. The frequencies of genetic polymorphisms often vary among different ethnic groups. In our study, the allele frequency for miR-423 rs6505162 A among the controls was 0.237, which is similar to that of Chinese population (0.200) and slightly higher than that of Japanese population (0.178). But the allele frequency is significantly lower than that of Caucasian population (0.529), European population (0.575) and African population (0.783) (http://www.ncbi.nlm.nih.gov/projecs/SNP). The incidence of NPC is significantly higher in China and Southeast Asian countries than that in Western countries [2]. It remains to be determined whether the differences of genotype and allele frequencies in miR-423 rs6505162 influence the risk of NPC in different ethnicities. No significant association was observed between the rs6505162 genotypes and clinical characteristics in NPC patients, which might be due to the relatively small sample size.

In conclusion, our data provide evidence, for the first time, that functional polymorphism of hsa-miR-423 rs6505162 might alter the susceptibility to NPC in Southern Chinese population. However, several limitations need to be addressed. Firstly, the sample sizes in our case-control study were relatively small. Secondly, the patients and controls were enrolled from the hospitals, so the inherent selection bias cannot be completely excluded. Thirdly, due to the limited information, subgroup analyses to other relevant factors such as EBV infection, diet, and family history have not been carried out, which restricted the power of our analyses. The current findings should be validated by more studies with large sample sizes, more populations from high-incidence areas such as Hong Kong, Guangxi, Hunan, and Singapore, and functional characterizations.

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Disclosure of conflict of interest

None.

Address correspondence to: Zhiwei He, Key Laboratory for Epigenetics of Dongguan City, Guangdong Medical University, Dongguan 523808, China. Tel: +86-0769-22896219; E-mail: zhiweihe688@yahoo.com

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