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Original Article

Hsa-miR-449a genetic variant is associated with risk of gastric cancer in a Chinese population

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Abstract: Gastric cancer (GC) is one of the most common malignancies and one of the major causes of cancer-related deaths worldwide. In the present study, we investigated the association between miR-449a rs112310158 SNP and GC risk. Our findings revealed that a variant GG genotype increased the risk of occurrence of GC compared to a wild type AA genotype (OR = 2.542, 95% CI: 1.304-4.954, *P* = 0.005). Specifically, the G allele reduced the risk of occurrence of cervical cancer in women compared to the A allele (OR = 1.279, 95% CI: 1.012-1.617, *P* = 0.043). In conclusion, our findings suggest that miR-449a rs112310158 is a genetic risk factor for GC.

Keywords: Gastric cancer, miR-449a, polymorphism

Introduction

Gastric cancer (GC) is one of the most common malignancies and one of the major causes of cancer-related deaths worldwide [1]. Studies found that gastric carcinogenesis is a complex, long-lasting multistep and multifactorial process [2]. Epidemiological studies have shown that environmental factors, including high salt intake, consumption of alcoholic and tobacco, and Helicobacter pylori (H. pylori) infection, contribute to the risk of gastric cancer [3]. However, not all the people exposed to the same environmental risk factors eventually developed gastric carcinoma, suggesting that host or genetic factors may also play a role in the occurrence of gastric cancer [4].

In recent years, many studies explored the potential association between single nucleotide polymorphisms (SNPs) and risk of gastric cancer [5]. Among the reported SNPs, MiRNA-related single nucleotide polymorphisms (miR-SNPs), which are defined as single-nucleotide polymorphisms (SNPs) in miRNA genes or the miRNA binding site are of great interest [6, 7]. MiRNAs are a class of conservative, small, single-strand, non-coding RNA molecules composed of around 22 nucleotides. MiRNAs regulate gene expression through degradation of target mRNAs or inhibition of translation by binding to complementary sequences in 3'-untranslated regions of messenger RNAs [8-10]. SNPs in miRNA genes or the miRNA binding site reportedly influence miRNAs expression, functions and individual susceptibility to cancers [11]. SNPs in miRNA may also play important roles in gastric cancer susceptibility through altering the expression or function of miRNAs, subsequently leading to aberrant expression of their target genes [12]. However, so far only very few studies have attempted to identify single nucleotide polymorphisms (SNPs) in miRNA genes which are associated with the occurrence of gastric cancer.

In the present study, we genotyped a mir-449a single nucleotide polymorphisms (rs112310158) in GC patients to evaluate the relationship of this genetic variant with risk for development of GC.

Materials and methods

Sample collection and DNA extraction

Blood samples were collected from GC patients who underwent GC resection at the Department of General Surgery at the Affiliated Hospital of Nantong University from 2012-2014. Blood samples were also collected from healthy volunteers without a history of any cancer. Ge-

Table 1. Clinical characteristics of the gastric cancer cases and control subjects

	Cases N = 448	Controls N = 452	OR (95% CI)	*P* value
Age, years				
< 55	179 (40.0%)	190 (42.0%)		
≥ 55	269 (60.0%)	262 (58.0)	1.090 (0.835-1.422)	0.542
Sex				
Female	148 (33.0%)	159 (35.2%)		
Male	300 (67.0%)	293 (64.8%)	1.100 (0.835-1.449)	0.527
Cancer history in the first relatives				
No	428 (95.5%)	447 (98.9%)		
Yes	20 (4.5%)	5 (1.1%)	4.178 (1.554-11.231)	0.002
Alcohol drinkers				
Never	202 (45.1%)	276 (61.6%)		
Ever	246 (54.9%)	176 (38.9%)	1.910 (1.465-2.490)	0.000
Cigarette smokers				
Never	260 (58.0%)	294 (65.0%)		
Ever	188 (42.0%)	158 (35.0%)	1.345 (1.028-1.761)	0.034
Helicobacter pylori				
Negative	207 (46.2%)	249		
Positive	241 (53.8%)	203	1.428 (1.098-1.857)	0.009
Histologic type				
Squamous cell carcinoma	382 (85.3%)			
Adenocarcinomas	36 (8.0%)			
Adenosquamous carcinoma	30 (6.7%)			

nomic DNA was immediately extracted using QIAamp® Blood Mini Kit (QIAGEN Inc., Valencia, CA, USA) according to the manufacturers instruction and was stored at -20°C. This study was supervised and approved by the Human Tissue Research Committee of the Affiliated Hospital of Nantong University. Written consent was obtained from all the participants enrolled in this study.

Genotyping

The genotypes of rs112310158 were detected by polymerase-chain reaction (PCR)-direct sequencing assay. The 178-bp DNA fragment containing the polymorphic site was amplified using two primers (forward 5'-AGTGGCTTGCTTCATAGCAGAA-3', and reverse 5'-GTGCTCTGGATACCTGTGTGT-3'). Polymerase chain reaction was performed in a total volume of 25 μl reaction mixture containing 50 ng of genomic DNA, 0.2 μl Taq DNA Polymerase (Takara, Dalian, China), 0.5 μl dNTP, 0.5 μl each primer, 2 μl 10 × Buffer and ddH_2O. The polymerase chain reaction profile consisted of an initial melting step at 94°C for 3 minutes followed by 40 cycles of 94°C for 45 seconds, 60°C for 45 seconds and 72°C for 30 seconds, and an additional extension 72°C for 7 minutes in a thermal cycler (CFX-96, Bio-Rad). Purified products were sequenced on an ABI Prism 3730 × l sequencer (Applied Biosystems, Foster City, CA, USA) using BigDye Terminator Sequencing Standards.

Real-time quantitative polymerase chain reaction (RT-qPCR) of miR-449a

Total RNA was extracted from human tissues using Trizol reagent (Invitrogen, NY, USA) according to the manufacturer's instructions. miR-449a quantification was performed using TaqMan MicroRNA Assays (Applied Biosystems, Foster City, USA). RT primer and TaqMan probe of miR-449a (Applied Biosystems, Foster City, USA) were used for PCR analysis on a Bio-Rad CFX-96 (Bio-Rad, Hercules, CA, USA) in accordance with the manufacturer's protocol. RNU6B, as an internal control, was used as endogenous controls to normalize miR-449a.

Immunohistochemical analysis

Immunohistochemistry (IHC) was performed as described previously [13].

Table 2. Genotype frequencies of the miR-449a rs112310158 polymorphism among GC cases and controls and their association with GC risk

Genotypes	Cases No. (%)	Controls No. (%)	OR (95% CI)	*P* value
rs112310158				
	N = 448	N = 452		
AA	288 (64.3%)	307 (67.9%)		
AG	129 (28.8%)	132 (29.2%)	1.042 (0.779-1.394)	0.824
GG	31 (6.9%)	13 (2.9%)	2.542 (1.304-4.954)	0.005
A Allele	705 (78.7%)	746 (82.5%)		
G Allele	191 (21.3%)	158 (17.5%)	1.279 (1.012-1.617)	0.043

Table 3. Increased genetic susceptibility to GC in subjects over 55 years of age at the miR-449a rs112310158 polymorphism locus

Genotypes	Cases No. (%)	Controls No. (%)	OR (95% CI)	*P* value
rs112310158				
	N = 269	N = 262		
AA	169 (62.8%)	178 (67.9%)		
AG	77 (28.6%)	77 (29.4%)	1.053 (0.721-1.540)	0.847
GG	23 (8.6%)	7 (2.7%)	3.461 (1.447-8.276)	0.004
A Allele	415 (77.1%)	433 (82.6%)		
G Allele	123 (22.9%)	91 (17.4%)	1.410 (1.042-1.909)	0.027

Statistical analyses

Statistical analyses were performed using the statistical package SPSS, version 12.0 (Chicago, IL, USA). All values are presented as mean ± standard deviation. Genotype and allelic frequencies were compared between the two groups by the Chi-Square test. The associations of genotypes of rs112310158 with risk of gastric cancer were estimated by calculating the odds ratios (OR) and their 95% confidence intervals (CIs) from multivariate logistic regression models. A *P* value less than 0.05 was considered the cutoff for statistical significance.

Results

Participant information

Characteristics of GC patients and healthy volunteers are summarized in **Table 1**. This case-control study enrolled 448 GC patients and 452 healthy volunteers. There was no statistically significant in the age and sex distributions between GC patients and healthy volunteers (*P* > 0.05). When comparing Clinical characteristics between GC cases and volunteers groups, GC cases were more likely to be cigarette smokers (OR = 1.345, 95% CI = 1.028-1.761) and alcohol drinkers (OR = 1.910, 95% CI = 1.465-2.490), have cancer history in the first relatives (OR = 4.178, 95% CI = 1.554-11.231), and have higher infection rate of Helicobacter pylori (OR = 1.428, 95% CI = 1.098-1.857).

Association between miR-449a rs112310158 SNP and GC risk

Genotyping at miR-449a rs112310158 in control patients revealed the following distribution: 72.6% CC, 24.5% CG, and 9.2% GG (**Table 2**). A chi-squared test showed that, in healthy women, the distribution of genotypes at the miR-449a rs112310158 locus was in accordance with Hardy-Weinberg equilibrium (χ^2 = 0.069, *P* = 0.792), indicating that these subjects are representative of the general population.

Unconditional logistic regression analysis showed that a variant GG genotype increased the risk of occurrence of GC compared to a wild type AA genotype (OR = 2.542, 95% CI: 1.304-4.954, *P* = 0.005); an AG genotype did not confer a statistically significant change in risk. Specifically, the G allele reduced the risk of occurrence of cervical cancer in women compared to the A allele (OR = 1.279, 95% CI: 1.012-1.617, *P* = 0.043) (**Table 2**). Further, Stratified analysis of the age showed that subjects over 55 years of age, the G allele further increased the risk of GC compared to the A allele (OR = 3.461, 95% CI: 1.447-8.276, *P* = 0.004) (**Table 3**).

Correlation between miR-449a rs112310158 polymorphism and risk of being infected with Helicobacter pylori

Unconditional logistic regression analysis showed that the GG genotype reduced the risk of occurrence of Helicobacter pylori infection in women compared to the AA genotype (OR = 2.683, 95% CI: 1.376-5.229); The G allele was associated with reduced risk of occurrence of

Table 4. The miR-449a rs112310158 polymorphism genotype is correlated with Helicobacter pylori infection

	Helicobacter pylori positive	Helicobacter pylori negative	OR (95% CI)	*P* value
rs112310158				
	N = 444	N = 456		
AA	280	315		
AG	133	128	1.169 (0.874-1.564)	0.653
GG	31	13	2.683 (1.376-5.229)	0.003
A Allele	693	758		
G Allele	195	154	1.385 (1.095-1.752)	0.007

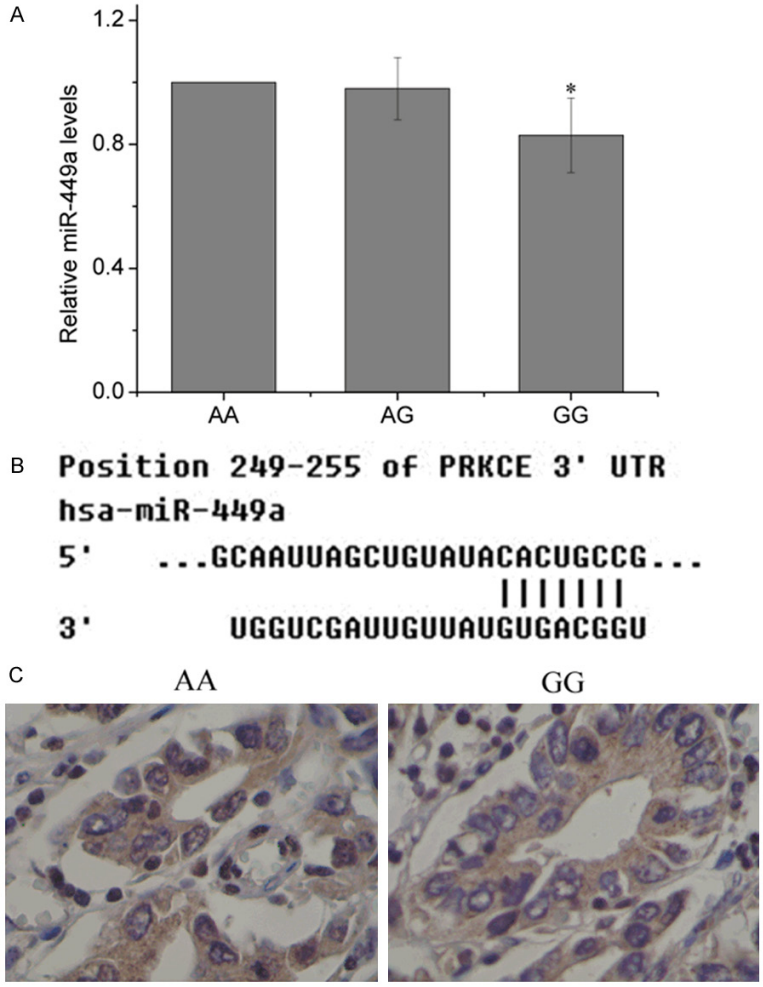

Figure 1. Correlation between rs112310158 and the expression of miR-449a and its target gene. A: The expression of miR-449a was detected by QRT-PCR. B: miR-449a target gene was searched using the public database TargetScan (http://www.targetscan.org). C: PRKCE expression was detected by *i*mmunohistochemical staining. *P < 0.05.

Helicobacter pylori infection compared to the A allele (OR = 1.385, 95% CI: 1.095-1.752) (**Table 4**).

Correlation between rs112310158 and the expression of miR-449a and its target gene

Using fluorescence quantitative PCR assay, we detected the expression of miR-449a in GC tissues from various genotypes. We found the miR-449a levels were significantly lower in patients with GG genotype than that in patients with AA genotype (**Figure 1A**). Furthermore, miR-449a target gene was searched using the public database TargetScan (http://www.targetscan.org), and protein kinase C, epsilon (PRKCE) that possessed a critically conserved binding site for miR-449a was selected for further study (**Figure 1B**). We further evaluated the clinical significance of PRKCE expression in patient various genotypes. Immunohistochemical staining (**Figure 1C**) demonstrated that PRKCE expression was higher in GC patients with GG genotype compared with that in patients with AA genotype.

Discussion

In the present study, we investigated the association between miR-449a rs112310158 SNP and GC risk. Our findings revealed that miR-449a rs112310158 GG genotype was significantly related to increased risk of GC compared with those carrying the AA genotypes. Furthermore, we found miR-449a rs112310158 polymorphism was associated with the expression of miR-449a and its target gene (PRKCE).

microRNA-449a (miR-449a) is a miRNA that has been previously identified in several types of cancers. miR-449a, which functions as a

cancer suppressor gene, is closely associated with a great variety of cancer, including lung [14], liver [15] and gastric [16] cancer; prostatic carcinoma [17, 18]; ovarian [19]; and breast [20] and bladder [21] carcinoma. In normal conditions, miR-449a was found to be strongly expressed in testis, lung and trachea tissue [22]. Down-regulation of miR-449a has been detected in several cancers including GC. Li et al reported that miR-449a was downregulated in gastric cancer cell lines and gastric cancer tissues [23]. They found miR-449a/E2F3 axis plays an important role in proliferation and apoptosis in gastric cancer. Similarly, Hu et al. provided evidence that miR-449a could modulate cell cycle and apoptosis through regulating cyclin D1 and BCL2 expression in SGC7901 cells [24]. However, the exact role of miR-449a in gastric cancer remains unknown. In the present study, we found miR-449a rs112310158 SNP was associated with GC risk in a Chinese population. A variant GG genotype of rs112310158 increased the risk of occurrence of GC compared to a wild type AA genotype (OR = 2.542, 95% CI: 1.304-4.954, P = 0.005).

Kinases are central mediators of signal transduction and an important class among them is protein kinase C (PKC) which constitutes 2% of the human kinome [25]. PKC is a series of structurally related serine/threonine kinases that are classified as conventional PKC, novel PKC and atypical PKC based on their structural properties and responsiveness to second messengers [26]. Since their discovery as receptors for tumor-promoting phorbol esters, PKCs have been intensively studied for their contribution to cancer [27]. Common processes regulated by PKCs include cell survival, proliferation, apoptosis, migration and invasion [28]. PKCε is the first isozyme that was shown to possess oncogenic functions and is emerging as an undisputed tumor promoter. PKCε is overexpressed in various tumor types and is associated with different processes related to cancer development namely, cell transformation, cell survival, cell proliferation, EMT, cytoskeletal reorganization, extracellular matrix (ECM) rearrangement, disruption of cell-cell contacts, cell motility, stem cell properties and therapy resistance [29]. In this study, we found protein kinase C epsilon possessed a critically conserved binding site for miR-449a and may be a direct target gene of miR-449a. We found the miR-449a levels were significantly lower in patients with GG genotype than that in patients with AA genotype. Furthermore, we found PRKCE expression was higher in GC patients with GG genotype compared with that in patients with AA genotype. These findings indicated that miR-449a rs112310158 may influence the risk of GC through modulating PRKCE expression.

In conclusion, our findings suggest that miR-449a rs112310158 is a genetic risk factor for GC. Further larger-scale and well-designed clinical studies are warranted to validate this finding.

Disclosure of conflict of interest

None.

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