Biomarkers, 2010; 15(7): 614–618

informa
healthcare

ORIGINAL ARTICLE

A functional variation in pre-microRNA-196a is associated with susceptibility of esophageal squamous cell carcinoma risk in Chinese Han

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Abstract

MicroRNAs (miRNAs) are small, non-protein-coding RNAs that function as tumour suppressors or oncogenes. A single nucleotide change in the sequence of pre-miRNA can affect miRNA expression, so single-nucleotide polymorphisms (SNPs) in pre-miRNA may be biomarkers for biomedical applications. In this study, we performed a genetic association study between the SNP (rs11614913) in pre-miRNA-196a and esophageal squamous cell carcinoma (ESCC) susceptibility in a case–control study. We found that the homozygote CC of this SNP increased the risk of ESCC compared with the homozygote TT and the risk was more evident among smokers than non-smokers. Therefore, this functional SNP may be a biomarker for ESCC outcome.

Key words: *SNP; miRNA-196a; esophageal cancer*

Introduction

Esophageal cancer is the sixth leading cause of cancer-associated death worldwide and the incidence rate has increased significantly in the past two decades (Brown et al. 2008, Jemal et al. 2008). Overall survival of this cancer is less than 10%, and the 5-year survival rate is 20–40% after surgery (Jemal et al. 2008). Therefore, the early detection of this disease is very important for the patients. There are two main forms of esophageal cancer: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EA) (Hoffman et al. 2009). Tobacco smoking is the major risk factor for ESCC, whereas reflux disease is the major risk factor for EA (Yu et al. 1988). ESCC is the main form found in China. There are many genes involved in esophageal cancer genesis such as *FRA-1*, *ID1*, *CDC25B*, *MET* (Hu et al. 2001). The newly found non-coding small RNAs are also involved in this cancer, such as microRNA (miRNA)-373 (Lee et al. 2009). Genetic and epigenetic factors might play an important role in esophageal cancer aetiology (Vallbohmer et al. 2009).

In 1993, Victor Ambros discovered a miRNA, lin-4, which affected development in *Caenorhabditis elegans* (Lee et al. 1993). Subsequently, more than 700 miRNAs have been identified in humans to date. miRNAs are small (about 22 nt) non-protein-coding RNAs that function as gene regulators by targeting mRNAs at the 3′-untranslated region, causing the mRNA cleavage or translational repression (Bartel 2004). Therefore, miRNAs regulate gene expression negatively. They have been found to regulate over 30% of mRNA gene expression and play important roles in cell growth, apoptosis, haematopoietic lineage differentiation and differentiation (Ambros 2004, Kloosterman & Plasterk 2006). miRNA alteration is an early event during cancer development

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(Received 18 May 2010; revised 26 June 2010; accepted 28 June 2010)

ISSN 1354-750X print/ISSN 1366-5804 online © 2010 Informa UK, Ltd.
DOI: 10.3109/1354750X.2010.505299

and miRNA polymorphism is a candidate biomarker for early diagnosis. In the past few years, many pre-miRNA single-nucleotide polymorphisms (SNPs) have been implicated in human cancers, including breast cancer, lung cancer and thyroid cancer (Hoffman et al. 2009, Jazdzewski et al. 2009), but there is no research on ESCC.

Maru et al. demonstrated that miRNA-196a is a potential marker of progression during Barrett's esophagus (BE) to EA and they found that the miRNA-196a expression level was much higher in EA than in normal esophageal cancer (Maru et al. 2009). Because a single nucleotide change in the pre-miRNA can affect the miRNA expression (Brennecke et al. 2005), SNPs in pre-miRNA may be candidates for biomedical applications. Previous studies found that the SNP in pre-miRNA-196a was associated with breast cancer and non-small-cell lung cancer in the Chinese population (Hu et al. 2009, Tian et al. 2009). However, whether or not this SNP (rs11614913) has an influence on ESCC risk in the Chinese population remains largely unknown. In this study, we hypothesized that the SNP in pre-miR-196a might affect the susceptibility to ESCC. To test this hypothesis, we performed an association study between this SNP and ESCC in a case–control study. To our knowledge, this is the first study that evaluates the effects of genetic polymorphism in pre-miRNA genes on the risk of ESCC in a Chinese Han population.

Methods

Study population

All subjects were genetically unrelated Han Chinese and were from Chongqing City of southwest China. The patients with ESCC were histopathologically diagnosed and confirmed at the Southwest Hospital, the first affiliated hospital of the Third Military Medical University. The exclusion criteria included previous cancer, metastasized cancer and previous radiotherapy or chemotherapy. The controls were healthy individuals who participated in a physical examination in Chongqing who had no history of cancer and were frequency matched to the cases on age, sex and residential area. Informed consent was obtained from the subjects, and the study was performed with the approval of the ethics committee of Third Military Medical University; the interviewers collected the information about the demographic data and environmental exposure history using a questionnaire. An individual who smoked >100 cigarettes in his or her lifetime was defined an ever-smoker. Former smokers were those who had quit smoking at least 1 year before diagnosis (for cases) or enrolment into this study (for controls). Recent quitters (RQ) were those who had quit within 1 year of diagnosis (for cases) or enrolment into this study (for controls). Pack-year for smokers ((cigarettes per day/20) x years smoked) was calculated to indicate the cumulative smoking dose. After interview, about 5 ml venous blood was collected from each participant.

PCR amplification

The genomic DNA was extracted by the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. Polymerase chain reaction (PCR) was run in a 20-μl volume containing 1 μl genomic DNA, 2× master mix (Tiangen, Beijing, China) and 0.25 μl of each primer. The PCR protocol entailed 5 min at 95°C; 30 cycles of 1 min at 95°C, 30 s at 62°C and 30 s at 72°C; and a 5-min final extension at 72°C. Primer sequences are sense: 5′-AGTCCTTAGGGAGGTTGTGGG-3′; antisense: 5′-AGGGATTGGGATAGGTTGAGA-3′.

Genotyping

We used the SNaPshot assay to achieve the genotype of the SNP (rs11614913 T/C). The SNaPshot PCR was run in a 10-μl volume containing 3 μl PCR products, 5 μl SNaPshot multiplex kit (ABI, Applied Biosystems, Foster City, CA, USA), 1 μl primer. The PCR protocol entailed 25 cycles of 10 s at 96°C, 5 s at 50°C and 30 s at 60°C. The primer is 5′-AACTCGGCAACAAGAAACTG-3′. Electrophorese samples and Liz120 (ABI) using Genetic Analyzer 3130 (ABI). The data were analysed using the 4.0 Genemapper software (ABI).

Statistical analysis

Differences between cases and controls were evaluated by the Student's *t*-test for continuous variables and χ^2 test for categorical variables. The associations between rs11614913 and esophageal cancer risk were estimated by the odds ratios (ORs) and 95% confidence intervals (CIs) using the general genetic model. The potential gene–environment interaction was evaluated by logistic regression analysis and tested by comparing the changes in deviance (-2 log likelihood) between the models of main effects with or without the interaction term. The χ^2 test for Hardy–Weinberg equilibrium was applied to the SNP among controls. All the statistical analyses were done with Statistical Package for the Social Sciences software (SPSS 13.0; SPSS, Chicago, IL, USA).

Results

The characteristics of the selected cases and controls are summarized in Table 1. There were 458 ESCC cases

Table 1. Characteristics of esophageal squamous cell carcinoma cases and controls.

Variables	Controls, *n* (%)	Cases, *n* (%)	*p*-Value
Age (years), mean ± SD	59.9 ± 6.74	58.9 ± 8.45	0.79
Sex			
Male	357 (73.0)	337 (73.6)	0.842
Female	132 (27.0)	121 (26.4)	
Total	489	458	
Smoking status			
Never	235 (48.1)	157 (34.3)	**<0.01**
Former	18 (3.7)	14 (3.1)	
Current and RQ	236 (48.2)	287 (62.6)	
Total	489	458	
Drink alcohol			
Yes	247 (50.5)	210 (45.8)	0.15
No	242 (49.5)	248 (54.2)	
Total	489	458	
Pack-years of smoking			
0	235 (48.1)	157 (34.3)	**<0.01**
1–30	116 (23.7)	98 (21.4)	
>30	138 (28.2)	203 (44.3)	
Stage			
I		7 (1.5)	
IIa		383 (83.6)	
IIb		44 (9.6)	
III		15 (3.3)	
IV		9 (2.0)	

RQ, recent quitter.
Significant *p*-values (<0.05) are in bold.

and 489 cancer-free controls in this case–control study. There were no significant differences between cases and controls in the terms of the age ($p = 0.79$), sex ($p = 0.842$) and drinking alcohol ($p = 0.15$), suggesting that our frequency matching of the demographic characteristics was satisfactory. As is well known, tobacco smoking is a major risk factor for ESCC and we found that about 65.7% of cases were smokers, which was much higher than for the controls (51.9%, $p < 0.01$). From the pack-years of smoking analysis, we found that the cases were more frequently heavy smokers (>30 pack-years) than the controls (44.3% vs 28.2%; $p < 0.01$). There were seven (1.5%) stage I (T1N0M0), 383 (83.6%) stage IIa (T2-3N0M0), 44 (9.6%) stage IIb (T1-2N1M0), 15 (3.3%) stage III (T3N1M0 or T4NanyM0) and nine (2.0%) stage IV (TanyNanyM1) esophageal cancer cases.

The association of the SNP with the ESCC risk is listed in Table 2. The observed genotype frequency for this polymorphism was in agreement with that expected under the Hardy–Weinberg equilibrium among the controls ($p = 0.60$). In the general genetic model, we found that the CC homozygous genotype of the SNP (rs11614913) located in miRNA-196a-2 was significantly associated with increased esophageal cancer risk compared with the wild-type homozygous TT and heterozygous CT (OR 1.35; CI 1.02–1.78). To examine whether the genetic variant was modified by epidemiological factors, we performed stratified analyses based on gender, smoking and age by logistic regression analysis and we found no significant interactions between this genotype and gender, age and differentiation.

To evaluate the gene–smoking interactions, we performed stratification analyses by smoking status using logistic regression analysis and we found that the risk of esophageal cancer associated with rs11614913 CC genotype was more evident among smokers (OR 3.27; 95% CI 1.97–5.40) than non-smokers (OR 1.70; 95% CI 0.99–1.70). In addition, we performed stratification analyses according to TNM stage to examine the association between rs11614913 genotype and ESCC risk. We found there is no association of this SNP with the ESCC stages ($p = 0.23$) (Table 4).

Discussion

In this association study of ESCC in a Chinese Han population, we found that the variant genotype CC was significantly increased risk of esophageal cancer compare with TT (OR 2.67; 95% CI 1.77–4.04). That is to say the C allele is a risk factor in ESCC. A previous study found that the same polymorphism (rs11614913) was significantly associated with breast cancer in China (Hu et al. 2009). Maru et al. found that miRNA-196a was a potential marker of progression during BE to EA and they found that the expression levels of miRNA-196a at different stages in patients were different (Maru et al. 2009). However the mechanism is ambiguous. The effect of the SNP rs11614913 on the expression of mature miRNA-196a may arise from the change of G:C to G:T in the stem region of the precursor. Whole gene expression profiling studies revealed that some genes decreased during progression of BE to EA, such as the *ANXA1* gene (Kimchi et al. 2005, Selaru et al. 2002). Interestingly, many of these genes were potential targets of miRNA-196a *in silico*. These studies were all on EA. From the present study we concluded that this SNP in pre-miRNA-196a might be a biomarker of ESCC diagnosis in Chinese people.

In the stratification analyses in the present study we found that the risk of esophageal cancer associated with the rs11614913 CC genotype was more evident among smokers than non-smokers (Table 3). As is well known, smoking is a major risk factor for ESCC. The observed difference may result from the interaction between the polymorphism and the lifestyle during carcinogenesis.

In this study we found that the variant genotype CC of rs11614913 was associated with the increased risk of esophageal cancer. Interestingly, the same C allele, which is a rare allele in the Chinese population, was associated with risk in breast cancer, non-small-cell

Table 2. Main effect of pre-microRNA-196a2 single-nucleotide polymorphisms on esophageal squamous cell carcinoma.

Genotype	Cases (n=458), n (%)	Controls (n=489), n (%)	Odds ratio (95% confidence interval)[a]	p-Value[a]
TT	48 (10.5)	111 (21.4)	1.00 (reference)	
CT	262 (57.2)	250 (53.9)	2.42 (1.66–3.54)	<0.01
CC	148 (32.3)	128 (24.7)	2.67 (1.77–4.04)	<0.01
TT/CT	310 (67.7)	361 (75.3)	1.00 (reference)	
CC	148 (32.3)	128 (24.7)	1.35 (1.02–1.78)	0.038

[a]Adjusted for age, sex and pack-years of smoking.

Table 3. Stratification analyses for rs11614913 according to smoking status.

Genotype	Smoking status	Cases(n=458), n (%)	Controls(n=489), n (%)	p-Value[a]	Odds ratio[a](95% confidence interval)
TT	No	23 (5.0)	53 (10.8)		Reference
CT	No	91 (19.9)	116 (23.7)	0.04	1.81 (1.03–3.17)
CC	No	43 (9.4)	66 (13.5)	0.2	1.50 (0.81–2.80)
CT/CC	No	134 (29.3)	182 (37.2)	0.05	1.70 (0.99–2.90)
TT	Yes	25 (5.5)	58 (11.9)		Reference
CT	Yes	171 (37.3)	134 (27.4)	<0.01	2.96 (1.76–2.98)
CC	Yes	105 (22.9)	62 (12.7)	<0.01	3.93 (2.23–6.91)
CT/CC	Yes	276 (60.2)	196 (40.1)	<0.01	3.27 (1.97–5.40)

[a]Odds ratios and p-values were calculated by multivariate unconditional logistic regression, adjustment for age and sex.

Table 4. Stratification analyses for rs11614913 in patients with esophageal squamous cell carcinoma according to TNM stage.

Genotype	I/II(n=434), n (%)	III/IV(n=24), n (%)	p-Value[a]	Odds ratio[a](95% confidence interval)
TT	45 (10.4)	3 (12.5)		Reference
CT	246 (56.7)	16 (66.7)	0.97	0.98 (0.27–3.49)
CC	143 (32.9)	5 (20.8)	0.38	0.52 (0.12–2.28)

[a]Adjusted for age, sex and pack-years of smoking.

lung cancer and ESCC in the Chinese population. So the C allele and T allele may have different functions. The miRNAs are regulators by targeting to the mRNAs and several *HOX* gene family members are found to be targets of miRNA-196a (Yekta et al. 2004). In 2009, a study found that the four *HOX* family genes, *HOXB2*, *HOXB3*, *HOXB5*, *HOXC13*, were significantly down-regulated after treatment with pre-miRNA-196a-C, but pre-miRNA-196a-T (Hoffman et al. 2009). Several studies reported that the *HOX* genes were deregulated in esophageal cancer and these genes possibly participate in the development of esophageal cancer (Chen et al. 2005, Takahashi et al. 2007). Furthermore, this study found a role for this SNP in esophageal cancer diagnosis; similar studies are warranted to determine whether or not there are prognostic implications for this SNP among esophageal cancer patients and the sample size should be enlarged. Also, additional studies are needed to clarify the association of the polymorphism with esophageal cancer in diverse ethnic populations, because polymorphisms often vary between ethnic groups.

In conclusion, the variant genotype CC of rs11614913 was associated with increased risk of ESCC, and the functional SNP rs11614913 could contribute to ESCC susceptibility in a Chinese Han population and this may be a biomarker for ESCC diagnosis.

Acknowledgements

The authors would like to acknowledge Yong Fu and Jun Wu in the Department of Thoracic and Cardiac Surgery, Southwest Hospital, Third Military Medical University.

Declaration of interest

This work was supported by the National Natural Science Foundation of China (no. 30971603 and no. 30872549) and Natural Science Foundation Project of CQ (CSTC 2009BA5013).

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