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A functional varient in microRNA-146a is associated with risk of esophageal squamous cell carcinoma in Chinese Han

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Abstract MicroRNAs are a new class of non-protein-coding, small RNAs that function as tumor suppressors or oncogenes. They participate in diverse biological pathways and function as gene regulators. A G>C polymorphism (rs2910164), which is located in the sequence of miR-146a precursor, results in a change from G:U to C:U in its stem region. However, it remains largely unknown whether this single nucleotide polymorphism (SNP) may alter esophageal squamous cell carcinoma (ESCC) susceptibility. In the current study, we evaluated association between rs2910164 and ESCC susceptibility in a case–control study of 444 sporadic ESCC patients and 468 matched cancer-free controls in a Chinese Han population. Compared with rs2910164 variant genotype CC, genotype GG was associated with increased risk of ESCC (Odds Ratio, 2.39, 95% Confidence Interval, 1.36–4.20). In the smokers, the risk of rs2910164 GG genotype was more notable (Odds Ratio, 3.17, 95% Confidence Interval, 1.71–4.46). In the stratification analyses, we also found there was a strong correlation between rs2910164 C/G variant and the clinical TNM stage ($P < 0.01$). These findings suggest that this functional SNP in pre-miR-146a could contribute to ESCC susceptibility and clinical outcome.

Keywords Esophageal squamous cell carcinoma (ESCC) · MicroRNA (miRNA) · Single nucleotide polymorphism (SNP) · Clinical outcome

Abbreviations

ESCC	Esophageal squamous cell carcinoma
miRNA	MicroRNA
SNP	Single nucleotide polymorphism

Introduction

Esophageal cancer can be divided into esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma, which is one of the the leading cause of cancer-related deaths in the world and the incidence rate has been increasing significantly in the last two decades [1, 2]. Compared with European and American nations, there is higher incidence for ESCC in China. ESCC is an extremely fatal disease. In spite of major advancement in cancer treatment, prognosis is still poor. Overall survival is less than 10%, and the 5-year survival rate is 20–40% after surgery [3]. So the early detection of this disease is very important for the patients.

Recently, many studies suggested that microRNAs (miRNAs) were involved in ESCC carcinogenesis. MicroRNA (miRNA) are 21- to 24-nucleotide-long small non-coding RNA gene products that regulate gene expression by binding with target mRNAs at the 3′ untranslated region (3′UTR), leading to mRNA cleavage or translational

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repression. It has been suggested that miRNAs regulate a wide range of biological processes, including differentiation, cell proliferation and apoptosis [4]. Thus the loss- or gain-of-function of specific miRNAs were thought to be key events in the tumorgenesis.

Single nucleotide polymorphisms (SNPs) or mutations in miRNA sequence have been found to affect the processing and target selection of human miRNAs. A G>C polymorphism (rs2910164) in the miR-146a precursor, which results in a change from G:U to C:U in its stem region, has been found to contribute to breast cancer, papillary thyroid carcinoma, prostate cancer and hepatocellular carcinoma susceptibility [5–8]. It's interesting to propose this polymorphism in pre-miR-146a gene sequence may be a good candidate cancer biomarker.

To date, there is no data about association between rs2910164 and ESCC. In the present study, we performed genotyping analyses for this SNP, then evaluated the association with susceptibility and clinic stage of ESCC in a case–control study of 444 esophageal cancer cases and 468 cancer-free controls in a Chinese Han population.

Materials and methods

Subjects

The case–control study consisted of 444 ESCC patients and 468 cancer-free controls. All subjects were genetically unrelated Chinese Han and from Chongqing City and the surrounding regions. The ESCC patients were histopathologically diagnosed and recruited between July 2005 and Auguest 2009 at the Southwest Hospital (Chongqing, China), without the restrictions of age and sex. The exclusion criteria included previous cancer, metastasized cancer and family history of cancer. All patients were enrolled with a response rate of 94%. The TNM stage of esophageal cancer at the time of diagnosis was classified into stage I (T1N0M0), stage IIa (T2–3N0M0), stage IIb(T1–2N1M0), stage III (T3N1M0 or T4NanyM0), and stage IV (TanyNanyM1) according to the American Joint Committee on Cancer (AJCC) TNM Classification of Carcinoma of the Esophagus Primary Tumor. Histopathologic classifications were based on postoperative histopathologic examination. Cancer-free controls, having no history or family history of cancer and other genetic disease, were recruited from individuals who visited the same hospital for physical examination between 2005 and 2009, and were frequency matched to the cases on age, gender, and residential area (urban or countryside).

After the written informed consent was obtained from each subject, a structured questionnaire was administered to collect information on demographic data and environmental exposure history. Those who had smoked <1 cigarette per day and <1 year in their lifetime were defined as non-smokers, otherwise they were considered as smokers. The smokers who quit for > 1 year were considered former smokers. Pack-years smoked [(cigarettes per day/20) * years smoked] were calculated to indicate the cumulative smoking dose. 5 mL venous blood sample was collected from each participant after interview, drawn in Vacutainer tubes containing EDTA and stored at −80°C. The study was performed with the approval of the ethical committee of Third Military Medical University.

Genotyping

Extraction of Genomic DNA was performed using Wizard Genomic DNA PuriWcation Kit (Promega, USA) according to the protocol. The quantity and quality of DNA was determined by using NANODROP 1000 (Theremo, USA).

The SNP (rs2910164) in the pre-miR-146a was described previously, which was common (minor allele frequency >0.10) in Chinese Han populations. We used SNPshot assay (ABI, USA) to achieve the genotypes of the SNP (rs2910164 G/C). Firstly it was amplified by using PCR, which was carried out in a 20 μl volume containing 1 μl genomic DNA, 10μl master mix (Tiangen,Beijing), 0.5 μl of each primer and 8μl water. Conditions for PCR were: an initial denaturation step for 5 min at 95°C, then 30 s at 95°C, 30 s at 61°C, followed by a step for 30 s at 72°C for 30 cycles, then a final extension for 5 min at 72°C. The primer pair was: a forward primer 5′-ccacccacatcagccttcc-3′ and a reverse primer 5′-ctgccttctgtctccagtcttc-3′. After purification by using FastAP Thermosensitive Alkaline Phosphatase and Exonuclease I (Fermentas, USA), the PCR products were mixed and used as templet in the SNPshot PCR reaction. The SNaPshot PCR was run in a 10 μl volume containing 3 μl PCR products, 5 μl SnaPshot multiplex kit (ABI, USA), 1 μl primer and 1 μl water. The PCR condition contained 25 cycles of 10 s at 96°C,5 s at 50°C and 30 s at 60°C.The primer was 5′-ttttttcagctgaagaactgaatttca-3′. Then the reaction product was purified by adding 1U FastAP Thermosensitive Alkaline Phosphatase (Fermentas, USA) for 1 h at 37°C and diluted in Hi-Di Formamide (ABI, USA) with Liz120 (ABI, USA). Electrophoresis was carried out on the ABI 3130 Genetic Analyzer(ABI, USA), the data was analyzed by the software Genescan 4.0 (ABI, USA). 10% of the samples were randomly selected to do sequencing, and the results were also 100% concordant.

Statistical analysis

Hardy–Weinberg analysis was performed by comparing the observed and expected genotype frequencies using

Chi-square test. Differences in demographic variables and selected variables were evaluated by using the Chi-square test. The odds ratios (OR) and 95% confidence interval (CI) were calculated by using an unconditional logistic regression model, for assessment the associations between pre-miRNA SNP and ESCC risk/ESCC clinic stage. All the statistical analyses were performed using SPSS13.0 software package (SPSS Company, Chicago, IL, USA). A probability level of 5% was considered as significant difference for all statistic analyses.

Results

Characteristics of the study population

The characteristics of the 444 esophageal cancer patients and 468 controls included in the analysis are summarized in Table 1. There were no statistically significant differences between cases and controls in terms of the frequency distribution of sex and age. As expected, smoking was a significant risk factor for ESCC. About 63.52% of the cases were current smokers, which were significantly higher than that of the controls (49.36%, $P < 0.01$), and ESCC cases are more frequent to be heavy smokers (> 30 pack-years) than the controls (47.75 vs. 28.85%, $P < 0.01$). For all 444 ESCC cases, there were 7(1.58%), 377(84.91%), 37(8.33%), 15(33.78%) and 8(1.80%) patients for clinic I, IIa, IIb, III and IV, respectively. Most of the ESCC cases were well- or moderate-differentiated (50.68 and 40.32% respectively).

Table 1 Distribution of selected variables in esophageal cancer cases and controls

Characteristics	ESCC patients *n* (%)	Controls *n* (%)	*P*-value
Total	444	468	
Age (years)			0.89
<60	238(53.60)	253(54.06)	
≥60	206(46.40)	215(45.94)	
Sex			0.11
Male	315(70.95)	309(66.03)	
Female	129(29.05)	159(33.97)	
Smoking status			<0.01
Never	148(33.33)	221(47.22)	
Former	14(3.15)	16(3.42)	
Current	282(63.52)	231(49.36)	
Pack-years of smoking			<0.01
0	148(33.33)	221(47.22)	
1–30	84(18.92)	112(23.93)	
>30	212(47.75)	135(28.85)	
Differentiation			
Well	225(50.68)		
Moderate	179(40.32)		
Poor	40(9.00)		
TNM stage			
I	21(4.73)		
IIa	266(59.91)		
IIb	100(22.52)		
III	45(10.14)		
IV	12(2.70)		

Genotype distributions

All of the recruited samples were successfully genotyped for rs2910164. The observed genotype frequency was in agreement with that expected under the Hardy–Weinberg equilibrium in the controls ($P = 0.12$), providing no evidence of population stratification. As shown in Table 2, the allele and genotype distribution of the SNP in ESCC cases was statistically significantly different from that in control subjects ($P = 0.002$ and 0.004, respectively). The genotype frequencies for rs2910164 were 4.50% (CC), 42.80% (GC) and 52.70% (GG) in ESCC patients and 8.97% (CC), 47.00% (GC), and 44.03% (GG) in cancer-free controls. The association between the genotype and the risk of ESCC was further analyzed by using multivariate unconditional logistic regression, with adjustment for age, sex and smoke status. Rs2910164 allele G had a 1.38-fold increased risk of ESCC with statistical significance (95% Confidence Interval, 1.12–1.69). In the dominant genetic model, compared with rs2910164 homozygote CC, we also found its homozygote GG was associated with a significantly increased risk of esophageal cancer (Odds Ratio, 2.39, 95% Confidence Interval, 1.36–4.20).

Stratified analysis

To evaluate the gene-smoking interactions, we performed stratification analyses by smoking status and found that the risk of ESCC associated with rs2910164 GG genotype were slightly more evident among smokers (Odds Ratio, 3.17, 95% Confidence Interval, 1.71–4.46) than nonsmokers (Odds Ratio, 1.29, 95% Confidence Interval, 0.57–2.33) (Table 3). In addition, we performed stratification analyses according to age, sex, differentiation and TNM stage to examine the association between rs2910164 genotype and ESCC risk. We found no heterogeneity of the risk in the stratum of different age, sex and differentiation (data not shown). However, the distribution of allele of rs2910164 was significantly associated with the TNM stage of the ESCC patients ($P < 0.01$). Compared with rs2910164 CC genotype, GG genotype was significantly associated with advanced clinic TNM stage (Odds Ratio, 2.28, 95% Confidence Interval, 1.47–4.32) (Table 4).

Table 2 Frequency distribution of rs2910164 in ESCC patients and cancer-free controls and their associations with risk of esophageal cancer

		ESCC patients *n* (%)	Controls *n* (%)	*P*-value*	Odds ratio* (95% confidence interval)
Allele	C	230 (25.90)	304 (32.48)		Reference
	G	658 (74.10)	632 (67.52)	<0.01	1.38 (1.12–1.69)
Genotype	CC	20 (4.50)	42 (8.97)		Reference
	GC	190 (42.80)	220 (47.00)	0.04	1.81 (1.03–3.20)
	GG	234 (52.70)	206 (44.03)	<0.01	2.39 (1.36–4.20)
	GC/GG	424 (95.50)	426 (91.03)	<0.01	2.09 (1.21–3.62)

* OR and *P*-values were calculated by multivariate unconditional logistic regression, adjustment for age, sex and smoke statues

Table 3 Stratification analyses for rs2910164 according to smoke statues

Genotype	Smoke statues	ESCC patients *n* (%)	Controls *n* (%)	*P*-value*	Odds ratio* (95% confidence interval)
CC	No	13 (8.78)	22 (9.95)		Reference
GC	No	73 (49.32)	111 (50.23)	0.78	1.12 (0.55–2.25)
GG	No	62 (41.89)	88 (39.82)	0.65	1.29 (0.57–2.33)
CC	Yes	7 (2.36)	20 (8.10)		Reference
GC	Yes	117 (39.53)	109 (44.13)	0.01	2.07 (1.25–3.54)
GG	Yes	172 (58.11)	118 (47.77)	<0.01	3.17 (1.71–4.46)

* OR and *P*-values were calculated by multivariate unconditional logistic regression, adjustment for age and sex

Table 4 Stratification analyses for rs2910164 in ESCC patients according to TNM stage

		Stage I/IIa *n* (%)	Stage IIb/III/IV *n* (%)	*P*-value *	Odds ratio* (95% confidence interval)
Allel	C	168 (29.27)	62 (19.75)		reference
	G	406 (70.73)	252 (80.25)	<0.01	1.58 (1.18–2.24)
Genotype	CC	17 (5.92)	3 (1.91)		reference
	GC	134 (46.69)	56 (35.67)	0.20	1.75 (0.68–2.52)
	GG	136 (47.39)	98 (62.42)	0.02	2.28 (1.47–4.32)
	GC/GG	270 (94.08)	154 (98.09)	0.06	2.13 (0.94–3.25)

* OR and *P*-values were calculated by multivariate unconditional logistic regression, adjustment for age, sex and smoke statues

Discussion

The association between SNPs in protein-coding genes and risk of cancer has been investigated extensively. However, few cancer association studies concerning the SNPs in miRNA genes have been reported. Here for the first time, we found variant genotype GG of miR-146a rs2910164 was associated with significantly increased risk of ESCC in the case–control study of esophageal cancer in Chinese Han. There were several association studies on this SNP in different types of cancers. Jazdzewski et al. reported that the GC genotype of miR-146a was associated with an increased risk for papillary thyroid carcinoma [6]. Shen et al. suggested that breast/ovarian cancer patients with C allele were prone to an earlier age of onset [5]. Xu T. et al. found that GG genotype was associated with the risk for hepatocellular carcinoma in male individuals [8]. Xu B. et al. found the CC genotype was associated with the reduced risk for prostate cancer [7]. However, there was no significant association between the cases and controls in Hu's and Tian's study in breast cancer and lung cancer respectively [9, 10]. These discrepancies may reflect the difference of cancer etiology and study populations.

Overexpression of miR-146a has been reported as a signature in breast, pancreatic and prostate cancers [11]. In a microarray-based miRNA expression analysis, miR-146a was found upregulated in ESCC tissues [12]. The effect of rs2910164 on the expression of mature miR-146a may be stemmed from the change of G:U to C:U in its stem region of the precursor. Furthermore, Jazdzewski et al. reported that papillary thyroid cells with GG genotype display higher level of endogenous miR-146a compared with those harboring CC genotype [6]. Conversely, Shen et al. found that the G-to-C variation resulted in increased production of mature miR-146a in breast cancer in cell model system [5]. These results suggest that sequence variations disturbing the

secondary structure of miRNA precursor may affect the maturation process of miRNA. However, the precise mechanisms regulating miRNA expression are not clear. Some other factors like location of the variants or the strength of the binding between nucleotides may contribute to this discrepancy. We need further investigations on the molecular mechanisms of how genetic variants might affect the mature miRNA expression.

Meanwhile, as Jazdzewski et al. reported, rs2910164 in pre-mir-146a could also affect target mRNA binding [6]. Two potential targets of miR-146a, including tumor necrosis factor receptor-associated factor 6 and interleukin-1 receptor-associated kinase 1, are key adapter molecules downstream of the Toll-like and cytokine receptors in the signaling pathways that play crucial roles in cell growth and immune recognition [13]. Furthermore, Jazdzewski et al. showed that GC heterozygotes differed from both GG and CC homozygotes by producing 3 mature microRNAs: one from the leading strand (miR-146a) and two from the passenger strand (miR-146a*G and miR-146a*C), each with its distinct set of target genes [14].

In stratified analysis in the present study, our results showed that GG genotype of miR-146a gene was associated with the risk for ESCC in the smokers ($P < 0.01$). However, the genotype distributions did not display significant difference between cases and controls in the no-smokers ($P = 0.89$). As we known, smoking is an independent risk factor for ESCC. The observed difference may result from the interaction between the polymorphism and the living habit during carcinogenesis.

It might be interesting to look at the links between rs2910164 and the clinic stage of ESCC. We found the distribution of allele of rs2910164 was significantly associated with the TNM stage of the ESCC patients ($P < 0.01$) and GG genotype was significantly associated with advanced clinic TNM stage. Because of the small sample size in the subgroups, these findings were primary and need to be validated in further studies. Our understanding of miRNA expression patterns and function in normal or neoplastic human cells is just starting to emerge.

Above all, the miRNA SNP may play an important role in esophageal cancer development and survival through influencing the expression/maturation of miRNAs and the binding of their mRNA targets. We concluded that this functional variant rs2910164 was a candidate biomarker of both esophageal cancer occurrence and survival. Further functional evaluation of the rs2910164, miR-146a miRNA and also its target mRNAs in esophageal cancer development, invasion, and metastasis are warranted.

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Conflict of interest We declare that there is no Conflict of interest.

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