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Original Article

Associations between polymorphisms in p53-related microRNA genes and overall survival of lung cancer in a Chinese non-smoking female population: a prospective cohort study

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Abstract: MicroRNAs (miRNAs) are suggested to be prognostic molecular markers of lung cancer. This study investigated the relationships between polymorphisms in three p53-related miRNAs including miR-26a-1, miR-34b/c, and miR-145 genes and overall survival (OS) of lung cancer, as well as the joint effect of miRNA polymorphisms in a Chinese non-smoking female population. A prospective cohort study including 295 diagnosed non-smoking female lung cancer patients was carried out, and patients were followed up until February 29th, 2016. Three miRNA polymorphisms (miR-26a-1 rs7372209, miR-34b/c rs4938723, and miR-145 rs353291) were analyzed. Univariate and multivariate Cox proportional hazards regression models were used. The TT genotype of miR-26a-1 rs7372209 may function as a risk factor of lung cancer OS (HR = 1.576, 95% CI = 1.022-2.430). The protective role of the CC genotype of miR-34b/c rs4938723 was observed (HR = 0.595, 95% CI = 0.373-0.949). No significant association was observed for the miR-145 rs353291 polymorphism. Compared with miR-26a-1 CC genotype and miR-34b/c CC genotype carriers, miR-26a-1 TT genotype and miR-34b/c TT genotype carriers had the highest death risk of lung cancer (HR = 3.476, 95% CI = 1.513-7.948). MiR-26a-1 rs7372209 and miR-34b/c rs4938723 polymorphisms may be associated with OS of lung cancer in Chinese non-smoking females, whereas miR-145 rs353291 was not significant associated with lung cancer prognosis. The combination of miR-26a-1 with miR-34b/c could act as a biomarker for lung cancer OS in this population.

Keywords: Lung cancer, microRNA, single nucleotide polymorphism, prognosis, overall survival, Chinese population

Introduction

Lung cancer is the most commonly diagnosed cancer and the leading cause of cancer death worldwide. In 2012, it was estimated that 1.6 million people died of lung cancer in the world, of which about 0.5 million were female [1]. In China, approximately 610.2 thousand people died of lung cancer in 2011, of which 177.8 thousand were female patients [2]. Although developments made in early diagnosis, surgical treatment, and adjuvant therapies based on chemotherapy, radiotherapy, and immunotherapy for lung cancer in recent years have made some significant benefits in patients' health outcomes, the overall prognosis of the patients is still poor with 5-year survival rates for lung cancer of only 17.8% at present [3]. Moreover, there are some significant differences in the health outcomes of lung cancer patients, even if they have the same conditions and treatments, which suggest that genetic factors are likely to affect the therapeutic effectiveness and prognosis of patients with lung cancer [4]. In Chinese non-smoking females, some previous studies have suggested that genetic background especially plays an indispensable role in the development of lung cancer [5-9]. The prevalence of cigarette smoking in women was as low as 2.7% displayed by Adult Tobacco Survey data in 2015, whereas it was more prevalent among men in China (52.1%) [10]. Smoking is

an established environmental risk factor for developing lung cancer, but the actual incidence and death of lung cancer are not matched which could probably be caused by smoking behavior among Chinese women. Therefore, it is necessary to find the molecular genetic markers of lung cancer in order provide precise treatment and improve the prognosis of Chinese non-smoking female patients.

In recent years, the study of microRNAs (miRNAs) as prognostic molecular markers of lung cancer has been rising gradually. MiRNAs are a subset of highly evolutionarily conserved short non-protein-coding RNAs with a length of 19-23 nucleotides [11]. miRNAs can induce the degradation or translation repression of the message RNAs (mRNAs) of protein-coding genes through completely or partially pairing with these mRNAs. It accounts for 1-5% of the human genome, and can regulate the expression of more than 30% of protein-coding genes [12]. Thus, miRNAs are involved in almost all important cellular biological processes, including proliferation, differentiation and apoptosis, and abnormalities in one of these processes may be associated with the susceptibility and prognosis of cancer [13]. In fact, it has been recognized that miRNAs can act as tumor suppressors or oncogenes. Further, previous studies have confirmed that abnormal expressions in some miRNAs are associated with the prognosis of lung cancer and that they can affect the recurrence of cancer, treatment efficacy, and survival time [14, 15]. In addition to this, single nucleotide polymorphisms (SNPs) in miRNA (miRNA-SNP) has been recognized for their role in cancer prognosis. MiRNA-SNPs have effects on the maturation of miRNAs or the affinity of miRNA binding to its target mRNAs, and may modify the expression level of cancer-related target genes, thereby affecting the prognosis of cancer patients [16]. Currently, accumulating studies have observed that several SNPs in miRNAs including miR-196a-2 rs11614913 [17], miR-30c-1 rs928505 [18], miR-423 rs6505162 [19], miR-146a s2910164 [20], miR-27a rs895819 [21], miR-5197 rs2042253 [22] and miR-499 rs3746444 [23] could be associated with lung cancer prognosis. Therefore, it is of great value for developing biomarkers of prognosis to explore the relationships between miRNA-SNPs and lung cancer.

As a multifunctional transcription factor, p53 is encoded by a tumor suppressor gene, which is one of the most commonly modulated genes in human cancer. In the past, p53 signaling networks were considered completely composed of protein-coding genes. The protein-coding genes regulated by p53 elicit various essential roles in regulating cellular processes including induction of cell senescence and apoptosis, and inhibition of metastasis [24]. Interestingly, these processes are also regulated and in some cases induced by some p53-regulated miRNAs [25]. In 2007, new members of the p53 signaling network, the miR-34 family, miR-34a and miR-34b/c, were reported to be directly regulated by p53, suggesting that there is an interaction between protein-coding genes and non-protein-coding RNAs in this critical tumor suppressor pathway [26]. Subsequently, many p53-related miRNAs were found to be involved in p53 classical biological functions, such as miR-26a-1 [27] and miR-145 [28]. In the last decade, the characterization of a number of miRNAs directly involved in p53 function and the cellular effects of these connections have been reported. However, to the best of our knowledge, the association between SNPs in the p53-related miRNAs mentioned above and lung cancer survival is seldom studied worldwide. Only one study conducted by Yoon et al. found that miR-26a-1 rs7372209 polymorphism did not affect the recurrence time of patients with non-small cell lung cancer (NSCLC) [20]. In other malignant tumors, such as esophageal cancer [29], gastric cancer [30], colorectal cancer [31, 32] and breast cancer [33], the exploration of the relationship between miR-26a-1 rs7372209 and prognosis showed inconsistent conclusions. Son et al. investigated the effect of miR-34b/c gene polymorphism on prognosis in 157 patients with hepatocellular carcinoma, and the results showed that after adjustment for age, gender and clinical characteristics, miR-34b/c rs4938723 were not found to have a significant relationship with 2-year survival rate [34]. Hu et al. found that the relationship between miR-145 rs353291 polymorphism and the prognosis of NSCLC was reversed in the different populations from a Phase II study [18]. In view of the inconsistency of the existing research results and the gaps of research in this filed, more research should be carried out to explore the relationship between SNPs in p53-related miRNAs and the overall survival (OS) of lung cancer.

Table 1. Baseline characteristics of the study subjects and their effects on OS

Characteristics	*n* (%)	MST (months)	Log-rank χ^2	*P*-value
Age (years)				
≤ 60	176 (59.7)	32.00	20.023	< 0.001
> 60	119 (40.3)	25.00		
Histological type				
Adenocarcinoma	230 (78.0)	29.00	4.022	0.134
Squamous carcinoma	48 (16.3)	24.00		
Others	17 (5.8)	21.00		
Clinical stage				
I	59 (20.0)	32.00	4.167	0.244
II	42 (14.2)	26.00		
III	169 (57.3)	28.00		
IV	25 (8.5)	28.00		
Chemotherapy				
No	19 (6.4)	31.00	1.173	0.279
Yes	276 (93.6)	28.00		
Surgery				
No	74 (25.1)	32.00	0.936	0.333
Yes	221 (74.9)	28.00		

OS = overall survival, MST = median survival time.

In the present study, we investigated the relationships of three SNPs in p53-related miRNAs (miR-26a-1 rs7372209, miR-34b/c rs4938723 and miR-145 rs353291) with the OS of lung cancer in a Chinese non-smoking female population.

Material and methods

Study design and sample

An ongoing molecular epidemiologic study of lung cancer was conducted in Shenyang, located in the northeast of China. The patients were recruited during February 2010 to December 2012 at Shenyang Northern Hospital, The First Affiliated Hospital of China Medical University, Liaoning Cancer Hospital & Institute. The inclusion criteria of patients were: (1) non-smoking females, (2) newly diagnosed with histologically confirmed lung cancer, (3) without chemotherapy or radiotherapy, (4) without other severe comorbidities. Patients with previous cancer or metastasized cancer from other cancers were excluded. All participants were from unrelated ethnic Han Chinese. Individuals with a total of 100 cigarettes in their life-time were defined as a smoker, otherwise they were considered a non-smoker. For each participant, 10 ml venous blood was drawn. Demographic information was collected by face-to-face interviews. Clinical data was obtained from clinical records. Specifically, demographic and clinical information included age, tobacco smoking, histological type, clinical stage, receipt of chemotherapy, and receipt of surgery.

In order to ensure a sufficient follow-up time for each participant, patients were followed up until February 29th, 2016. In the present study, death from lung cancer was defined as the outcome event. The death cause of each participant was collected based on data from Shenyang Center for Disease Control and Prevention (CDC) registry system. The date of death was confirmed based on Death Registry System of Shenyang Public Security Bureau. Finally, we collected complete information of 295 non-smoking female lung cancer patients. There were 18 (5.75%) patients lost to follow-up, and no statistical differences in demographic and clinical characteristics were found between lost to follow-up and follow-up patients.

The protocol of this study was approved by the institutional review board of China Medical University. This study was conducted in accordance with the amended Declaration of Helsinki. Informed consent was obtained from each participant or each participant's representative if direct consent could not be obtained.

SNP identification and genotyping

The Phenol-chloroform method was adopted to isolate genomic DNA samples from venous blood. The Applied Biosystems 7500 FAST Real-Time PCR System (Foster City, CA, USA) was used to perform SNP genotyping using Taqman1 allelic discrimination (Applied Biosystems, Foster City, CA) with primer probe set. In this study, 10% of samples were selected randomly, and the SNP genotyping of these

Table 2. Distribution of genotypes and the associations between the three SNPs and OS

SNP genotypes	*n* (%)	MST	*P*-value	Crude HR (95% CI)	*P*-value	HR[a] (95% CI)
miR-26a-1						
CC	148 (50.2)	31.00		1.000		1.000
CT	120 (40.7)	27.00	0.468	1.097 (0.854-1.411)	0.688	1.057 (0.819-1.365)
TT	27 (9.2)	28.00	0.127	1.389 (0.911-2.116)	0.033	1.597 (1.040-2.453)
Dominant model						
CT+TT vs. CC	147 (49.8)	27.00	0.272	1.142 (0.901-1.449)	0.313	1.132 (0.889-1.441)
Recessive model						
TT vs. CC+CT			0.110	1.384 (0.929-2.063)	0.020	1.623 (1.079-2.441)
miR-34b/c						
TT	174 (59.0)	28.00		1.000		1.000
TC	95 (32.2)	28.00	0.944	1.009 (0.780-1.305)	0.922	0.987 (0.757-1.287)
CC	26 (8.8)	35.00	0.073	0.664 (0.425-1.039)	0.041	0.620 (0.393-0.980)
Dominant model						
TC+CC vs. TT	121 (41.0)	29.00	0.597	0.937 (0.736-1.192)	0.423	0.903 (0.705-1.158)
Recessive model						
CC vs. TT+TC			0.065	0.662 (0.427-1.026)	0.038	0.623 (0.398-0.975)
miR-145						
AA	99 (33.6)	28.00		1.000		1.000
AG	150 (50.8)	28.00	0.880	1.021 (0.782-1.332)	0.621	0.933 (0.710-1.227)
GG	46 (15.6)	32.00	0.961	1.009 (0.705-1.445)	0.604	0.906 (0.625-1.315)
Dominant model						
AG+GG vs. AA	196 (66.4)	29.00	0.932	0.989 (0.768-1.274)	0.516	0.917 (0.705-1.191)
Recessive model						
GG vs. AA+AG			0.922	0.985 (0.721-1.345)	0.710	0.941 (0.684-1.295)

SNP = single nucleotide polymorphism, OS = overall survival, MST = median survival time, HR = hazard ratio, CI = confidence interval, HR[a] = HR adjusted by age, histological type, clinical stage, receipt of chemotherapy or surgery.

samples were performed a second time by two investigators separately. Genotyping results were checked for concordance by different investigators for quality control. Negative controls were included in each run of SNP genotyping.

Statistical analysis

Median survival time (MST) was compared by log-rank test between groups with different demographic and clinical characteristics. The Kaplan-Meier method and log-rank test were performed to evaluate the relationships of OS with miRNA-SNP genotypes. Hazard ratio (HR) and its 95% confidence interval (CI) for OS were estimated by univariate and multivariate Cox proportional hazards regression models. All data were analyzed by SPSS 22.0 (IBM, New York, NY, USA). A $P < 0.05$ (two-tailed) was considered statistically significant.

Results

Subject characteristics and OS

The demographic and clinical characteristics of subjects as well as their effects on OS are displayed in **Table 1**. Among the female lung cancer patients, the median of age was 57 years, ranging from 22 to 83 years, and median MST was 28 months. There were 230 adenocarcinomas and 48 squamous cell carcinomas in the pathologic types of lung cancer; 101 subjects at clinical stage I/II and 194 subjects at clinical stage III/IV; 276 subjects with receipt of chemotherapy; 221 subjects with receipt of surgery. The significant difference of MST was only observed in patients with different age. Compared with the MST of individuals ≤ 60 years (MST = 32 months), those who were > 60 years had a shorter OS (MST = 25 months) ($\chi^2 = 20.023$, $P < 0.001$). However, significant MST difference was not observed in patients

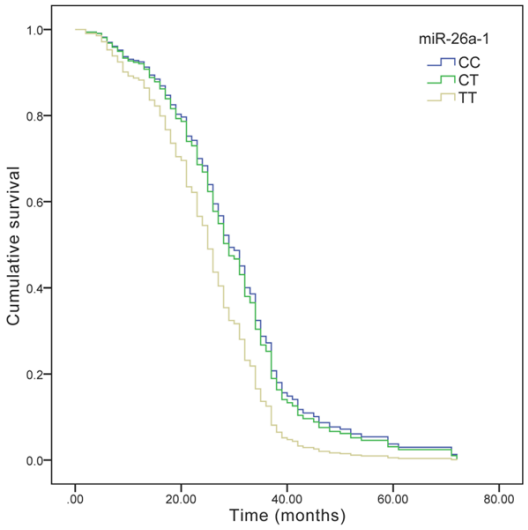

Figure 1. Survival curves for patients with different genotypes of miR-26a-1 rs7372209. Age, histological type, clinical stage and receipt of chemotherapy or surgery were adjusted.

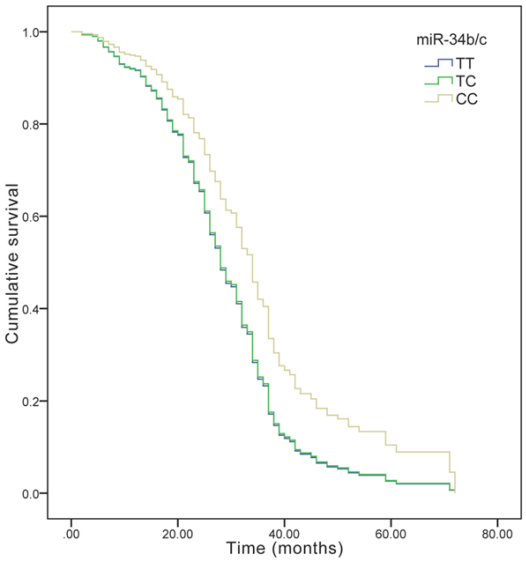

Figure 2. Survival curves for patients with different genotypes of miR-34b/c rs4938723. Age, histological type, clinical stage and receipt of chemotherapy or surgery were adjusted.

with different histological type and clinical stage, or in patients with or without receipt of chemotherapy or surgery.

Genetic polymorphisms and OS

The distribution of genotypes and the results of the relationships between the 3 SNPs and OS are summarized in **Table 2**. After adjusting age, pathological type, clinical stage, chemotherapy and operation, patients containing TT genotype of miR-26a-1 rs7372209 was independently associated with lung cancer survival (HR = 1.597, 95% CI = 1.040-2.453), who may have a shorter MST. The survival curves for patients with different genotypes of miR-26a-1 rs7372209 are shown in **Figure 1**. Patients containing CC genotype of miR-34b/c rs4938723 was independently associated with lung cancer survival (HR = 0.620, 95% CI = 0.393-0.980) as shown in **Figure 2** that was the survival curves for patients with different genotypes of miR-34b/c rs4938723, who may have a longer MST. Moreover, the recessive models of miR-26a1 rs7372209 (HR = 1.623, 95% CI = 1.079-2.441) and miR-34b/c rs4938723 (HR = 0.623, 95% CI = 0.398-0.975) were statistically significant. No statistically significant association was observed for the miR-145 rs353291 in this study.

Given the effects of patients' baseline characteristics and genetic polymorphisms on OS, multivariate Cox proportional hazards regression analysis was conducted. Results are summarized in **Table 3**. The TT genotype of miR-26a-1 rs7372209 may function as a risk factor of lung cancer survival (HR = 1.576, 95% CI = 1.022-2.430). A protective role of CC genotype of miR-34b/c rs4938723 was also observed (HR = 0.595, 95% CI = 0.373-0.949). Also, no significant association was observed for the miR-145 rs353291 polymorphism in the multivariate analysis.

Joint effect of the miRNA-SNPs on OS

The joint effect of miR-26a-1 rs7372209 and miR-34b/c rs4938723 on survival time is reported in **Table 4**. After adjusting for age, pathological type, clinical stage, chemotherapy, and operation, a slight joint effect of the two miRNA-SNPs was found. Compared with miR-26a-1 CC genotype and miR-34b/c CC genotype carriers, miR-26a-1 TT genotype and miR-34b/c TT genotype carriers had the highest death risk of lung cancer (HR = 3.476, 95% CI = 1.513-7.948).

Discussion

The present study investigated the effects of miR-26a-1 rs7372209, miR-34b/c rs4938723 and miR-145 rs353291 polymorphisms on the

Table 3. Associations between the three SNPs and OS in multivariate Cox proportional hazards regression analysis

Variables	HR	95% CI	*P*-value
Age (years)			
≤ 60	1.000		
> 60	1.760	1.368-2.265	< 0.001
Histological type			
Adenocarcinoma	1.000		
Squamous carcinoma	1.429	1.024-1.993	0.036
Others	1.483	0.804-2.783	0.207
Clinical stage			
I	1.000		
II	1.289	0.828-2.005	0.261
III	1.554	1.125-2.146	0.008
IV	1.591	0.910-2.783	0.103
Chemotherapy			
No	1.000		
Yes	1.627	0.956-2.769	0.073
Surgery			
No	1.000		
Yes	1.122	0.809-1.556	0.490
miR-26a-1			
CC	1.000		
CT	1.012	0.782-1.310	0.927
TT	1.576	1.022-2.430	0.040
miR-34b/c			
TT	1.000		
TC	0.992	0.757-1.298	0.952
CC	0.595	0.373-0.949	0.029
miR-145			
AA	1.000		
AG	0.901	0.681-1.192	0.466
GG	0.885	0.606-1.293	0.528

HR = hazard ratio, CI = confidence interval.

prognosis of patients with lung cancer in Chinese non-smoking females. The main results of our study showed that miR-26a-1 rs7372209 and miR-34b/c rs4938723 polymorphisms were significantly related to the risk of death in these patients. Moreover, a joint effect of the two p53-related miRNA-SNPs on the OS of these patients was also found in this study.

Research on the relationship between miR-34b/c rs4938723 and lung cancer prognosis has not found evidence yet. In hepatocellular carcinoma patients, there was no significant relationship between this polymorphism and 2-year survival rate [34]. In this study, we found that the CC genotype of miR-34b/c rs4938723 could improve the OS of Chinese non-smoking female patients. Landi et al. reported that after adjusting for age and clinical stage, lung squamous cell carcinoma patients with a high expression of miR-34c had a lower risk of death, and the expression of miR-34c had a significant relationship with the survival of surgical resection NSCLC patients [35]. Human miR-34b and miR-34c genes are localized on chromosome 11q23.1. MiR-34b/c is produced from its primary transcript (pri-miR-34b/c). Ectopic expression of miR-34 can produce the same biological effects as p53, inducing induction of cell cycle arrest, cell senescence, and apoptosis [36]. There are p53 binding sites in the promoter region of miR-34 family members that are expressed in a p53 dependent manner. Thus, miR-34 is a master regulator of tumor suppression putatively by regulating the expression of common miR-34 target genes such as MET, CDK4, CDK6, and E2F3 [37], which are closely related to the development and progression of tumors. For the miR-34b/c rs4938723 polymorphism, it was found that the C allele of miR-34b/c rs4938723 had a lower transcriptional activity than its T allele, which could lead to a reduction in miR-34b/c expression [38], and then increase the morbidity and mortality of cancer. To the best of our knowledge, this study was the first to explore the relationship between miR-34b/c rs4938723 and the prognosis of lung cancer, and show a significant association.

MiR-26a is a member of the miR-26 family, and mature miR-26a includes miR-26a-1 and miR-26a-2, which can influence the expression of multiple target genes that involved in cancer progression at the post-transcriptional level, such as EZH2, PTEN, SMAD1, and MTDH [39]. The results from previous studies of miR-26a expression in lung cancer are inconsistent; two of them found miR-26a showed a high expression in NSCLC [40, 41], whereas the other two

Table 4. Joint effect of miR-26a-1 rs7372209 and miR-34b/c rs4938723 on OS

Model	*n*	*P*-value	Crude HR (95% CI)	*P*-value	HR[a] (95% CI)
0	14		1.000		1.000
1	57	0.009	2.454 (1.246-4.833)	0.005	2.689 (1.349-5.362)
2	127	0.013	2.268 (1.184-4.344)	0.005	2.568 (1.327-4.968)
3	82	0.009	2.434 (1.253-4.725)	0.005	2.666 (1.354-5.250)
4	15	0.015	2.765 (1.222-6.256)	0.003	3.467 (1.513-7.948)

Model 0: CC of miR-26a-1 and miR-34b/c; Model 1: CT of miR-26a-1 and CC of miR-34b/c, or CC of miR-26a-1 and CT of miR-34b/c; Model 2: CT of miR-26a-1 and miR-34b/c, or CC of miR-26a-1 and TT of miR-34b/c, or TT of miR-26a-1 and CC of miR-34b/c; Model 3: CT of miR-26a-1 and TT of miR-34b/c, or TT of miR-26a-1 and CT of miR-34b/c; Model 4: TT of miR-26a-1 and miR-34b/c. OS = overall survival, HR = hazard ratio, CI = confidence interval, HR[a] = HR adjusted by age, histological type, clinical stage, receipt of chemotherapy or surgery.

studies reported a low expression of miR-26a in NSCLC [27, 42]. Human miR-26a-1 gene is localized on chromosome 3p22.2. Lezina et al. found that p53 can promote the expression of miR-26a-1 at the transcriptional level by directly acting on the -2000 to -1500 region of the miR-26a-1 gene [43]. Yoon et al. found that miR-26a-1 rs7372209 did not have effect on the recurrence time of surgical resection NSCLC patients [20]. Wu et al. indicated that miR-26a-1 rs7372209 was not associated with OS in patients with advanced esophageal cancer [29]. Jiao et al. found no significant association between the miR-26a-1 polymorphism and OS of breast cancer [33]. However, an opposite result was obtained in this study, and the association between miR-26a-1 rs7372209 and the OS of lung cancer in non-smoking female patients was revealed by univariate and multivariate analyses. Consistent with this result, Boni et al. reported that the miR-26a-1 polymorphism was associated with the prognosis of colorectal cancer, and the recurrence rate of patients with TT genotype was significantly shorter than that of patients with CC/CT genotype [31]. Also, in patients with advanced gastric cancer, miR-26a-1 polymorphism played an important role in the patients' prognosis, and the patients with the homozygous mutation had a lower overall survival rate [30]. In metastatic colon cancer patients treated with 5-fluorouracil and irinotecan, a significant association with tumor response and time to progression was found for miR-26a-1 rs7372209. The genotypes CC and CT were favorable when compared with the TT variant genotype [31].

Human miR-145 gene is located on chromosome 5q32. In a variety of tumors including lung cancer, as a tumor suppressor gene, miR-145 expression is low, and can inhibit cell proliferation, invasion, and metastasis, as well as increase cell apoptosis through the regulation of multiple target genes, such as c-Myc, IRS-1, and mucin1 [28]. Low expression of miR-145 can significantly reduce the OS of patients with lung cancer [44], and can shorten the time of lung cancer recurrence [45]. Hu et al. carried out a Phase II study in NSCLC patients [18], and the relationship between miR-145 rs353291 and the prognosis of NSCLC was found to be opposite in the different subjects from two stages. In this study, we found that miR-145 rs353291 was not related to the prognosis of Chinese non-smoking female patients with lung cancer. Thus, we provide new evidence about the relationship between miR-145 rs353291 and lung cancer prognosis in Chinese population. In view of the putative role of tumor suppression, other polymorphisms of miR-145 gene should be tentatively related to the prognosis of lung cancer in future studies.

Several limitations in this study should be elucidated. Firstly, the sample size was not large in the cohort study because we selected non-smoking females as subjects in order to control some confounding bias. Thus, the statistical power of this study could be limited. A larger sample size should be needed for further study. Secondly, this is a hospital-based study, which may have resulted in selection bias for the overall population. This was a multi-center study which enhances the reliability of the results. Thirdly, only three SNPs in p53-related miRNAs were investigated in the present study, and the other SNPs in the p53 pathway should been considered in future studies. In addition, functional assessment of the genetic variants identified in this study should be carried out through in vitro and in vivo experiments.

In conclusion, miR-26a-1 rs7372209 and miR-34b/c rs4938723 polymorphisms may be associated with the OS of lung cancer in Chin-

ese non-smoking females, whereas miR-145 rs353291 was not significantly associated with lung cancer prognosis. The combination of miR-26a1 with miR-34b/c could act as a biomarker for cancer survival in this population. These results still require confirmation by larger prospective studies.

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Disclosure of conflict of interest

None.

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