RESEARCH ARTICLE

Prognostic Implications for High Expression of MiR-25 in Lung Adenocarcinomas of Female Non-smokers

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Abstract

Background: Adenocarcinoma (ADC) is the most common histological type of lung cancer and its proportion is rising, especially in Asian non-smoking women. Recent studies suggest miR-25 may have diverse effects on the pathogenesis of different types of cancer. However, the role of miR-25 in lung cancer is still unknown. The aim of this study was to investigate the potential clinical value of miR-25 in non-smoking women with lung ADC. Patients and Methods: Quantitative RT-PCR was performed to evaluate the expression of miR-25 in 100 lung ADC tumor tissues and matched plasma samples and Pearson correlation tests were used to analyze the relationship between values. Associations of miR-25 expression with clinicopathological features were determined using the Student's t-test. To determine prognostic value, overall survival (OS) was evaluated using the Kaplan-Meier method. Univariate and multivariate analyses were performed using the Cox proportional hazard model. Results: Expression of miR-25 in tissue was found to be associated with lymph node metastasis (*P*=0.021) and disease stage (*P*=0.012). Moreover, high miR-25 expression was also associated with poorer overall survival of women with lung ADC (*P*=0.008). Conclusion: Tissue miR-25 expression may be associated with tumor progression and have prognostic implications in female lung ADC patients.

Keywords: miR-25 - lung adenocarcinoma (ADC) - prognosis - lymph node metastasis - disease stage

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Introduction

Lung cancer is the leading cause of cancer-associated death worldwide, and non-small cell lung cancer (NSCLC) accounts for approximately 80% of all lung cancers (Siegel et al., 2012). It is known that the majority of lung cancer cases are due to tobacco smoking. However, 10% to 20% of lung cancer involves people who have never smoked (Thun et al., 2008). Non-smoking lung adenocarcinoma (ADC) has been prominently increasing worldwide, and the incidence is particularly high in Asian women in which ADC is the most common histological type (Toh et al., 2006; Toh and Lim 2007; Wakelee et al., 2007). The clinical and pathological features, molecular markers, treatment response and prognosis of female non-smoking lung ADC are quite different from male NSCLC (Metayer et al., 2002; Rudin et al., 2009; Pallis and Syrigos 2013). Therefore, it is of great significance to identify potential biomarkers for predicting the progression and prognosis of lung ADC in women.

MicroRNA are small (18 to 24 nucleotides in length), single-stranded, endogenous non-coding RNAs that regulate gene expression. Mammalian miRNAs are generally encoded in the introns of pre-messenger RNA (mRNA) or the 3' untranslated region of mRNA. They suppress gene expression by binding to the complementary regions of mRNA, which either blocks translation or degrades mRNA through the RNA-induced silencing complex (Bartel, 2004). Recent studies have demonstrated that the alteration of miRNA regulation may play a pivotal role in the development and progression of cancer, and their aberrant expression may be implicated in carcinogenesis and tumor progression (Calin and Croce 2006; Volinia et al., 2006; Liu et al., 2012).

MiR-25 is a member of the miR-106b-25 cluster which consists of miR-106b, miR-25 and miR-93, and is located within intron 13 of the minichromosome maintenance complex component 7 (MCM7) gene on chromosome 7q22.1. The effects of miR-25 on cancer are complicated because it can be oncogenic or tumor suppressive in different types of cancer (Li 2009; Scapoli et al., 2010; Wang et al., 2010; Zhu et al., 2011a; Esposito et al., 2012; Suh et al., 2012; Zhang et al., 2012; Kim et al., 2013; Qiang et al., 2013; Wu et al., 2013). So far, the exact role of miR-25 and its clinical and prognostic significance in NSCLC is still unknown. In the present study, we used

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Table 1. Association Between miR-25 Expression and Clinicopathological Parameters of Patients with Lung Adenocarcinoma

Variable		miR-25 ($\bar{\chi}$±S)		miR-25 ($\bar{\chi}$±S)	
	N	in tissue	*P*-value	in plasma	*P*-value
Age at diagnosis (years)					
≤60	50	7.47±0.45	0.362	5.05±0.48	0.668
>60	50	7.39±0.40		5.00±0.36	
Family history of cancer[a]					
Yes	12	7.32±0.33	0.340	5.02±0.42	0.548
No	87	7.44±0.43		5.10±0.44	
History of lung disease					
Yes	8	7.42±0.34	0.937	4.77±0.15	0.119
No	92	7.43±0.43		5.05±0.43	
Disease stage					
I-II	57	7.34±0.41	0.012*	5.05±0.41	0.496
III-IV	43	7.56±0.41		4.99±0.44	
Tumor size					
≤3cm	55	7.49±0.43	0.180	5.07±0.42	0.322
>3cm	45	7.37±0.41		4.98±0.43	
Lymph node[a]					
N0	47	7.34±0.43	0.021*	5.08±0.44	0.195
N1/N2/N3	49	7.54±0.41		4.96±0.40	
Distant metastasis					
Yes	9	7.54±0.45	0.406	5.03±0.50	0.994
No	91	7.42±0.42		5.03±0.42	
EGFR mutation[a]					
Yes	43	7.55±0.39	0.002*	4.99±0.34	0.612
No	40	7.29±0.35		5.03±0.46	

[a]Total case number was less than 100 owing to missing data; *Indicate statistically significant (P<0.05)

real-time PCR to analyze the expression of miR-25 in both plasma and tissue samples of non-smoking women with lung ADC at Tianjin Medical University Cancer Institute and Hospital, aiming to investigate the potential clinical values of miR-25 in non-smoking women with lung ADC.

Materials and Methods

Patients and Samples

The study was approved by the medical ethics committee at Tianjin Medical University Cancer Institute and Hospital. All patients in the study were recruited from the Cancer Institute and Hospital between 2007 and 2009. Demographic features and tobacco use were collected using a structured questionnaire. We enrolled 100 female non-smoking patients with newly diagnosed and histologically confirmed lung ADC, and each patient provided a tumor tissue specimen and a matched blood sample for the study. Clinical information on histological type, tumor size, disease stage, lymph node involvement, distant metastasis at diagnosis, and treatments was extracted from medical records. All the patients were followed from surgery to August 20, 2013 though clinical visits and regular telephone contacts. Survival time was calculated from the date of diagnosis to the date of death or last follow-up (August 20, 2013).

A blood sample (10 ml) was collected from each patient using an EDTA vacutainer tube. Plasma was separated by centrifugation at 2,000 rpm for 20 min and then placed in a liquid nitrogen tank for storage until miRNA extraction. All matched tissue samples were histologically confirmed, and stored at -80°C until analysis.

RNA extraction and QRT-PCR

Total RNA was extracted from the flash frozen tissue samples using the TRizol reagents (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Total RNA was quantified with Nanodrop N-1000 (Agilent Biosystems TM, Santa Clara, CA, USA), and RNA quality was maintained through measurements of RNA purity by UV spectrophotometry and assessment of RNA quality and integrity. All RNA samples from tissue were diluted to 50 nanograms per microliter before analysis.

The miRNeasy Mini Kit (QiaGen, Hilden, Germany) was used to extract miRNA from the plasma of patients according to the manufacturer's instructions. Since no miRNA has been established as a 'house-keeping gene' in the plasma, we added 25 fmol of synthetic C. elegans miRNA cel-miR-39 (Johnson & Johnson, Skillman, NJ, USA) in each plasma sample as an external control to monitor the quality of our RNA extraction and analysis. Briefly, the reverse transcription (RT) reaction was carried out using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) both for tissue and plasma miRNA according to the instruction of the protocol. RT reaction was processed at 16°C for 30 min, 42°C for 30 min, and 85°C for 5 min. Following the RT, quantitative real-time PCR was performed in an ABI 7900 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) at 95°C for 10 min, followed by 40 cycles of 95°C for 15s and 60°C for one minute. The average quantification cycle (Cq) was calculated using the SDS 2.4 software (Applied Biosystems, Foster City, CA, USA). The average expression levels of miRNA in tissue and plasma were normalized with the levels of small nuclear RNU6B and cel-miR-39 respectively, using the $2^{-\Delta\Delta Cq}$ method.

Statistical Analysis

The program SPSS software package (version 16.0, SPSS Inc., Chicago, IL, USA) and Graphpad Prism 5.0 statistical software (Graphpad Software Inc., La Jolla, CA, USA) were utilized for all statistical analyses. RNA expression levels were calculated based on the formula of $\log_{10}2^{-\Delta\Delta Cq}$, and were analyzed as mean±SD. Pearson correlation coefficient test was used to analyze the correlation of miR-25 expression between paired plasma and tissue. Independent two-sample Student's t-test was used to analyze the associations between miR-25 expression and clinicopathological features of patients. The Kaplan-Meier method was used for survival analysis, and the differences in survival were estimated using the log-rank test. Prognostic factors were examined using the univariate and multivariate Cox proportional hazards regression models. Differences were considered statistically significant when the P value was less than 0.05.

Results

Patient characteristics and clinical features

A total of 100 female non-smoking patients with lung ADC were enrolled in the study. The median follow-up of these patients was 34 months (range 4-72 months), and

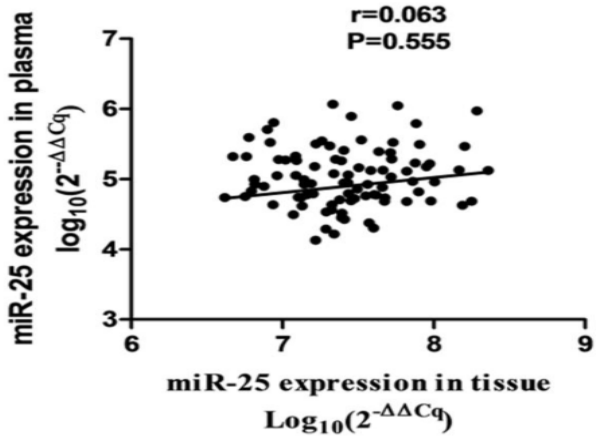

Figure 1. Correlation between the Expressions of miR-25 in Tissue and Plasma from Female Lung Adenocarcinoma Patients. There was no correlation between the expressions of miR-25 in tissue and circulation levels of ADC (Pearson r=0.063, *P*=0.555)

the mean age at diagnosis was 58.56±9.94 years, ranging between 34 and 81 years. The distributions of patient characteristics and clinical features were summarized in Table 1.

Correlation between the expressions of miR-25 in tissue and plasma

We analyzed the relationship of miR-25 expression between plasma and tissue levels of ADC, and found no correlation between the two (Pearson r=0.063, *P*=0.555, Figure 1).

Association between tissue miR-25 expression and clinicopathological features

We analyzed the association between miR-25 expression and clinicopathological features of ADC (including age, family history of cancer, history of previous lung disease, disease stage, tumor size, lymph node metastasis, and distant metastasis) to understand better the potential role of miR-25 in female lung ADC. Results showed that high expression of miR-25 in tissue was significantly associated with lymph node metastasis, advanced clinical stage, and EGFR mutation (*P*=0.021, 0.012 and 0.002 respectively; Table 1 and Figure 2), but no association was found between miR-25 expression in plasma and clinical-pathological features of lung ADC (Table 1).

Association between the expression of miR-25 and overall survival of lung cancer patients

We first analyzed patient survival outcomes using the Kaplan-Meier survival curves to assess the prognostic value of miR-25 expression in lung ADC. Patients were categorized as miR-25 high and low expression groups based on the mean expression. The 5-year cumulative probability of survival for patients with high miR-25 expression in tissue (34.7%) was significantly lower than those with low miR-25 expression (69.3%). The Kaplan-Meier survival curves showed that the patients with high miR-25 expression in tissue had shorter survival time than those with low miR-25 (Log-rank test, *P*=0.008, Figure 3A). As anticipated, patients with advanced disease stage (*P*=0.000, Figure 3B), regional lymph node metastasis (*P*=0.000, Figure 3C), and distant metastasis at diagnosis (*P*=0.004 Figure 3D) also had poor survival outcomes for

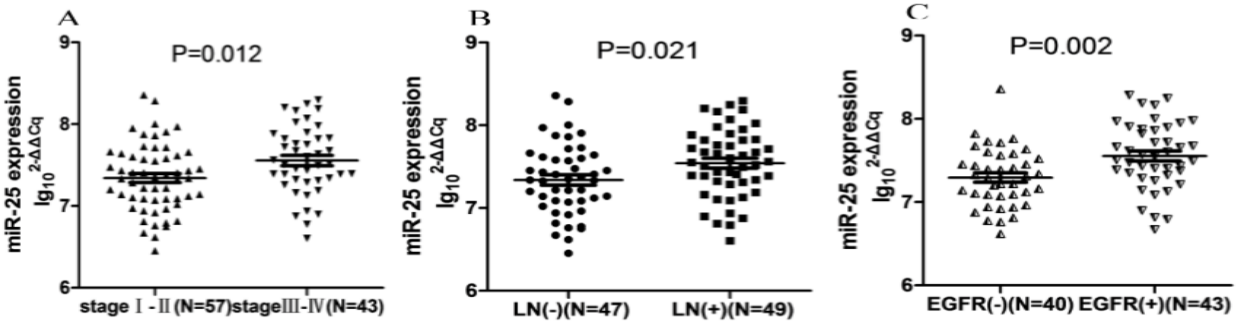

Figure 2. Levels of miR-25 in Different Groups. High expression of miR-25 in tissue was significantly associated with lymph node metastasis (*P*=0.021, 2A), advanced clinical stage (*P*=0.012, 2B), and EGFR mutation status (*P*=0.002, 2C)

Table 2. Univariate and Multivariate Analyses of Prognostic Factors in Female Lung Adenocarcinoma

Factors	Univariate analysis		Multivariate analysis	
	HR (95%Cl)	*P*-value	HR (95%Cl)	*P*-value
History of lung disease (Yes/No)	1.77 (0.62- 5.05)	0.286	-	-
Family history of cancer (Yes/No)	0.54 (0.17-1.76)	0.306	-	-
Lymph node metastasis (+/-)	3.33 (1.64 -6.79)	0.001*	1.58 (0.37-6.74)	0.536
Age (>60/≤60)	0.77 (0.40-1.49)	0.437	-	-
Disease stage (III-IV/I-II)	5.16 (2.46-10.79)	0.000*	2.90 (0.70-12.07)	0.144
Tumor size (>3cm/≤3cm)	1.23 (0.64-2.37)	0.538	-	-
Distant metastasis (Yes/No)	3.05 (1.39-6.73)	0.006*	1.30 (0.47-3.62)	0.612
miR-25 level in tissue (High/Low)	2.97 (1.46-6.03)	0.007*	2.34 (1.02-5.35)	0.044*
miR-25 level in plasma (High/Low)	0.78 (0.38-1.59)	0.498	-	-

*Indicate statistically significant (*P*<0.05)

Table 3. All these Genes Obtained from the miR-25-target Analysis were Categorized by Gene Ontology (GO) according Biological Process, Cellular Component and Molecular Function

Term	Gene Count	*P*-value	Gene name
Biological process			
Regulation of transcription	14	0.047	BTG2, GATAD2B, SERTAD3, SMAD7, SOX4, CREB1, RNF4, HIVEP1, LBX1, PHTF2, CIC, SUV420H1, TEF, ZNF287
Regulation of specific transcription fromRNA polymerase II promoter	3	0.034	SMAD7, CREB1, LBX1
Cell-matrix adhesion	3	0.031	BCL2L11, ITGAV, TSC1
Heart development	4	0.029	SMAD7, SOX4, LBX1, TSC1
Tube development	4	0.031	BCL2L11, GDF11, LBX1, TSC1
Kidney development	3	0.036	BCL2L11, GDF11, TSC1
Cellular component			
Cell leading edge	3	0.049	ITGA5, SLC12A5, TSC1
Molecular function			
Transcription factor activity	8	0.044	GATAD2B, SMAD7, SOX4, TEF, CREB1, RNF4, ZNF287, LBX1

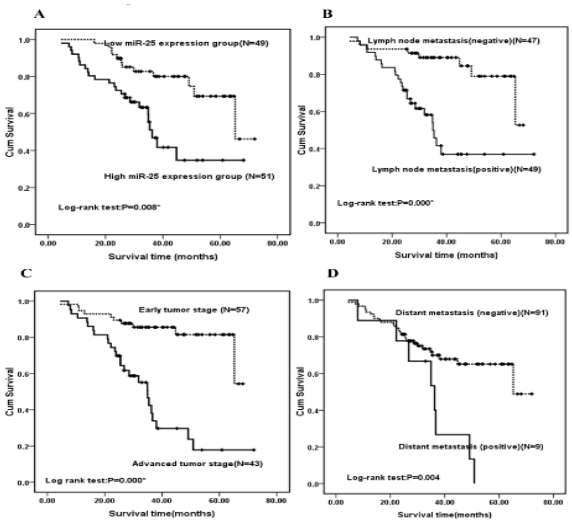

Figure 3. Kaplan–Meier Curves for Overall Survival in the 100 Lung Adenocarcinoma Patients. Poor survival outcomes of lung ADC patients were significantly associated with high miR-25 expression in tissue (*P*=0.008, 3A), advanced disease stage (*P*=0.000, 3B), regional lymph node metastasis (*P*=0.000, 3C), and distant metastasis at diagnosis (*P*=0.004 3D)

lung cancer.

Next, univariate and multivariate regression analyses were carried out to evaluate the factors for lung ADC prognosis. The results of univariate analysis showed that disease stage (*P*=0.000), lymph node metastasis (*P*=0.001), distant metastasis (*P*=0.006) and miR-25 expression in tissue (*P*=0.007) were significantly associated with survival (Table 2). Moreover, the multivariate Cox regression analysis indicated that miR-25 expression in tissue (*P*=0.044) was an independent prognostic factor for female patients with lung ADC. However, there was no significant association between plasma miR-25 level and lung cancer survival (Table 2).

Analysis of miR-25 predicted targets

Given the fact that the biological significance of miRNAs deregulation relies on the actions of their targets, we analyzed the predicted targets of the miR-25. The analysis was done using three databases: TargetScan 6.2 (http://www.targetscan.org/vert_61/), PicTar (http://pictar.mdc-berlin.de/) and miRanda (http://www.microrna.org/microrna /home.do), which are commonly used to predict the targets of miRNA. A total of 66 potential unique gene symbols targeted by miR-25 were predicted by all three algorithms, and these genes were categorized by GO analysis in the DAVID online database (http://david.abcc.ncifcrf.gov/tools.jsp) (Table 3). We identified seven biological processes which were statistically significant (*P*<0.05), including regulation of specific transcription from RNA polymerase II promoter, regulation of transcription, cell-matrix adhesion, system development and others. One cellular component of cell leading edge and one molecular function of transcription factor activity were also included.

Discussion

Lung cancer is the leading cause of cancer-related deaths worldwide. Although advances have been made recently in improving the diagnosis and treatment of lung cancer, the prognosis of the disease remains to be miserable (Raben et al., 2004). It is critical to identify new molecular markers for clinical management and prognosis prediction of lung cancer (Chen et al., 2013; Franchina et al., 2013; Yang et al., 2013). MiRNAs regulate gene expression at a post-transcriptional level and play a pivotal role in the regulation of cell development, metabolism, immunity, proliferation, differentiation, and apoptosis. Thus, they may serve as potential biomarkers to indicate different disease courses and outcomes. Wang et al have demonstrated that miR-138 suppresses ovarian cancer cell invasion and metastasis by targeting SRY-related high mobility group box 4 (SOX4) and hypoxia-inducible factor-1a (HIF-1a), and that miR-138low/SOX4high signature is associated with malignant phenotypes in ovarian cancer (Yeh et al., 2013). In the study of Garofalo et al, miR-34a and miR-34c were found to be down-regulated in lung tumors compared to normal tissues, and these miRNAs target platelet-derived growth factor receptor alpha and beta (PDGFR-αand PDGFR-β), cell surface tyrosine kinase receptors that induce proliferation, migration and invasion in lung cancer (Garofalo et al., 2013).

Interestingly, miR-25 is recently identified as both oncogenic miRNA (OncomiR) and tumor suppressor

miRNA (TSmiR) in the carcinogenesis of different types of cancer. For example, miR-25 is considered an OncomiR in hepatocellular carcinoma, gastric cancer, glioblastoma, oral carcinoma, breast cancer, esophageal adenocarcinoma, and ovarian cancer (Li 2009; Yang et al., 2009; Scapoli et al., 2010; Wang et al., 2010; Zhu et al., 2011a; Suh et al., 2012; Zhang et al., 2012; Kim et al., 2013; Wu et al., 2013), but TSmiR in colon cancer and thyroid carcinoma (Esposito et al., 2012; Qiang et al., 2013). However, the expression pattern of miR-25 in lung cancer and its relationships with the clinicopathological features and prognosis are still unclear.

In the present study, we first tried to address the miR-25 expression between the tissue and plasma samples from the same patients. Unfortunately, no correlation was found between the two types of samples. So far, few studies have investigated the relationship of miRNAs expression between the above two tissues in lung cancer. Shen et al examined 12 miRNAs levels in tissue and paired plasma samples of 28 NSCLC patients and found that 5 miRNAs (miR-21, 210, 182, 126 and 486-5p) in plasma samples had similar tendencies as in the tumors. However, seven miRNAs, including miR-139, 145, 205, 200b, 375, 429, and 708, did not display corresponding changes between plasma and tissue samples (Shen et al., 2011). Zhu et al. (2011b) reported serum levels of miR-96, but not miR-182 and miR-183, might correspond to the levels in tumors miR-96 in 76 NSCLC patients. To the best of our knowledge, this study is the first to report miR-25 expression in both tissue and plasma. The correspondence between circulating and local levels of different miRNAs remains to be elucidated.

Recently several studies reported that miR-25 was significantly related to lymph node metastasis and TNM stage in esophageal adenocarcinoma and gastric cancer (Zhu et al., 2011a; Kim et al., 2013; Wu et al., 2013). Similarly, our results also found that the up-regulation of miR-25 in lung tumor was significantly associated with advanced clinical stage, lymph node metastasis, and poor survival. Thus, miR-25 expression may be speculated to be involved in ADC pathogenesis and progression. Therefore, the molecular mechanisms underlying the role of this miRNA in cancer need to be addressed in future studies.

The biological significance of miRNA deregulation relies on the functions of their regulated mRNAs. We analyzed the predicted targets of miR-25 using the computational methods that have been widely used for prediction of miRNA targets. It has been shown that the common miRNA targets predicted by all three computational algorithms (miRanda, PicTar and TargetScan) are considered to be reliable and will be given the highest sensitivity (Lewis et al., 2003). Our results predicted a total of 66 unique gene symbols targeted by miR-25, and all of these genes were categorized into the biological processes, cellular component or molecular function. Among them, the transcription factor activity related genes, such as GATAD2B, SMAD7, SOX4, TEF, CREB1, RNF4, ZNF287 and LBX1, may probably be associated with tumor development and progress. SMAD7, one of the above target genes, has already been verified by an in vitro experiment. Wang et al., identified SMAD7 as a direct target of miR-25 in colon cancer. They found that miR-25 could directly interact with the SMAD7 3'UTR to suppress its mRNA and protein expression. Moreover, reintroduction of SMAD7 could partly reverse the inhibitory effects of miR-25 on cell proliferation and migration, which suggests that the antitumor effect of miR-25 partly acts through repressing SMAD7 in colon cancer cells (Qiang et al., 2013). In addition to the computational predicted targets, CDH1, which encodes the epithelial cell adhesion molecule E-cadherin, a transmembrane glycoprotein that forms the core of adherens junctions between adjacent epithelial cells, was illustrated to be suppressed by miR-25, and thus could promote cell migration and invasion of esophageal squamous cell carcinoma (ESCC) (Xu et al., 2012). Since the reduction or loss of E-cadherin expression has been characterized as the epithelial-mesenchymal transition (EMT) process and is associated with lymph node metastasis and an unfavorable prognosis in NSCLC (Myong, 2004), a similar process may probably be explained for our finding that high miR-25 expression was associated with the advanced stage, lymph node metastasis and poor survival of lung ADC. Moreover, Bim has been identified as a direct target of miR-25 in ovarian cancer. Zhu et al., demonstrated that down-regulation of miR-25 induced apoptosis caused by repressing expression of Bim (Zhang et al., 2012). In addition, miR-25 could also target P57 through the 3'UTR in gastric cancer (Min et al., 2013), and thereby involved in cell apoptosis, transcriptional regulation, cell fate determination, cell migration and cytoskeletal dynamics (Besson et al., 2008). Ectopic expression of miR-25 results in activation of CDK2 and facilitation of G1/S phase transition.

Furthermore, our results indicated that miR-25 was significantly up-regulated in EGFR-positive group compared with the EGFR-negative group. This result is in agreement with the finding of Dacic's study which investigated the miRNA expression in lung adenocarcinomas with different oncogenic mutations, including EGFR-positive, KRAS-positive and EGFR/KRAS-negative tumors. Their results showed miR-25 was up-regulated in EGFR-positive group (Dacic et al., 2010). It is known that activating EGFR mutations are common in patients with ADC histological type who are female never smokers and Asian ethnicity (Lynch et al., 2004; Pao et al., 2004). Recently, Milella et al suggested that MEK inhibition may regulate PTEN expression by selectively interfering with the expression of miR-25 (Ciuffreda et al., 2012) , which has recently been shown to regulate PTEN levels in human tumors and contribute to experimental tumorigenesis *in vivo* (Poliseno et al., 2010). Since the tumor suppressor gene PTEN (phosphatase and tensin homolog), which encodes a phosphoinositide phosphatase, is a downstream molecule in the EGFR pathway (Manning and Cantley, 2007; Salmena et al., 2008; Ciuffreda, 2009), it is plausible that the prognosis of ADC patients with EGFR mutation may be improved by using TKI in combination with a miR-25 blocker in the future.

In conclusions, our study found no correlation of the miR-25 expression between tumor tissue and circulating

levels in female non-smoking lung ADC. However, the up-regulation of tissue miR-25 expression was significantly associated with regional lymph node metastasis, advanced disease stage, and poor prognosis of female non-smoking patients with lung ADC. These findings suggest that miR-25 may serve as a new marker for clinical management and prognosis prediction of lung cancer, especially for those female patients with EGFR mutation. However, the precise mechanism of miR-25 in NSCLC tumorigenesis and progression still needs further investigation.

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