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MicroRNA-141 Is a Biomarker for Progression of Squamous Cell Carcinoma and Adenocarcinoma of the Lung: Clinical Analysis of 125 Patients

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Lung cancer is the most common malignant tumor worldwide. MicroRNA has become an ideal biomarker for cancer diagnosis, prognosis and therapy. The relationship between microRNA-141 and non-small cell lung cancer (NSCLC) is contradictory. Thus, in current study, we aimed to investigate the level of microRNA-141 in NSCLC tissues and to evaluate its potential clinical value. This study enrolled 125 NSCLC patients (75 males and 50 females) with a median age of 61 years (range, 23-90 years). NSCLC patients included 23 squamous cell carcinomas (SCCs), 101 adenocarcinomas (ADCs) and 1 large cell carcinoma. The expression level of microRNA-141 was significantly higher in NSCLC tissues than in adjacent lung tissues ($P < 0.001$), detected by real time RT-PCR. Receiver operating characteristic (ROC) exhibited a moderate diagnostic value of microRNA-141 for NSCLC with the area under curve of 0.707. The microRNA-141 expression increased with the larger tumor size ($P = 0.002$), lymph node metastasis ($P = 0.018$) and advanced stage ($P = 0.022$) in NSCLC patients. For subgroup analysis, microRNA-141 expression in SCC was correlated with tumor size ($r = 0.490$, $P = 0.018$), and in ADC, microRNA-141 level was positively associated with tumor size ($r = 0.222$, $P = 0.026$), lymph node metastasis ($r = 0.242$, $P = 0.015$) and TNM stage ($r = 0.210$, $P = 0.035$). Furthermore, univariate analysis revealed that the expression of microRNA-141 was an independent prognostic indicator of ADC. In conclusion, microRNA-141 is a potential biomarker for the molecular diagnosis and risk stratification of NSCLC.

Keywords: adenocarcinoma; microRNA-141; non-small cell lung cancer; squamous cell carcinoma; survival
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Introduction

Lung cancer, as one of the leading causes of cancer-related death worldwide, has resulted in rising death rates, with approximate 1.4 million deaths per year all over the world (Jemal et al. 2011; De Mello et al. 2013). Lung cancer is divided into two major pathological categories: small cell lung cancer (SCLC, 13%) and non-small cell lung cancer (NSCLC, 87%) (DeSantis et al. 2014). NSCLC, including squamous cell carcinoma (SCC), adenocarcinoma (ADC), adenosquamous cell carcinoma and large cell carcinoma (LCC), accounts for roughly 80-87% of all lung cancer cases (Jemal et al. 2011), while the occurrence of SCLC is much lower. Furthermore, the treatment patterns were different between SCLC and NSCLC patients, especially the chemotherapy (Kuwabara et al. 2009), as well as molecular targeted therapy (Lauro et al. 2014; Asai et al. 2014). The most effective treatment for early NSCLC is surgical resection (Gazala et al. 2013). However, nearly 2/3 of patients with NSCLC are diagnosed at an advanced or metastatic stage of disease. In the circumstances, the most beneficial treatment is chemotherapy and/or concurrent administration of chemotherapy and radiation. In addition, usage of specific inhibitors (such as gefitinib, erlotinib, afatinib, crizotinib) is effective for those patients with a mutation in the epidermal growth factor receptor (EGFR) gene or rearrangement of the anaplastic lymphoma kinase (ALK) gene (Chen et al. 2013a; Gridelli et al. 2014). Nevertheless, on

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account of late stage diagnosis and high frequency of drug resistance, the 5-year survival rate in patients is only 17% in the United States (Siegel et al. 2014; DeSantis et al. 2014). The unfavorable outcome is due to the relatively inadequate understanding of the molecular mechanisms involved in lung carcinogenesis (Brothers et al. 2013). Thus, there is an urgent requirement to discover new early detection markers and novel therapeutic targets of NSCLC. However, the treatment of SCLC is still given priority to chemotherapy (Asai et al. 2014).

MicroRNAs are a class of small, endogenous, non-coding RNAs that modify the expression of protein-coding genes by binding the sequence to the 3′-untranslated region (3′-UTR) of target mRNA, leading to the repression of translation or degradation of the mRNA (Guz et al. 2014; Kang and Lee 2014; Oom et al. 2014; Ritchie and Rasko 2014). MicroRNAs play critical roles in tumorigenesis, tumor cell proliferation, migration, invasion, metastasis and angiogenesis (Iuliano et al. 2013; Di Leva et al. 2014). A large number of microRNAs have been aberrantly found in NSCLC, and many of them contribute to NSCLC development and progression (Chen et al. 2013b; Xia et al. 2014b; Xia et al. 2014a). The role of microRNAs may be altered between NSCLC (Hu et al. 2008) and SCLC (Liu and Chen 2011), thus we only focus on NSCLC. There are many factors influencing the prognosis of lung SCC and ADC. For example, high expression levels of MutS homologue 2 (MSH2) had a better outcome in SCC. However, increased MSH2 expression was a poor prognostic factor in ADC patients (Vageli et al. 2012). Vascular invasion was an independent prognostic factor in ADC, whereas it was not significant for SCC patients (Usui et al. 2013). Patients with SCC with the interleukin-10 non-ATA haplotype had poorer overall survival and relapse-free survival than those with the ATA haplotype. However, interleukin-10 haplotype was not directly correlated with the clinical outcome of patients with ADC (Wang et al. 2013). MicroRNA-662, -192 and -192* were confirmed as independent prognostic factors in SCC patients, while there was no similar findings in ADC (Skrzypski et al. 2014).

Has-microRNA-141, a member of an evolutionarily conserved family of microRNAs, locates in chromosome 12 and belongs to the same cluster as has-miR-200c. Growing evidence has indicated that abnormal expression/function of microRNA-141 was associated with tumorigenesis and carcinoma progression of various human cancers (Zhou et al. 2014; Liu et al. 2014; Chen et al. 2014b). MicroRNA-141, as a tumor suppressor, was down-regulated in numerous cancer types, such as gastric cancer (Chen et al. 2014a), hepatocellular carcinoma (Wu et al. 2014) and pancreatic cancer (Zhao et al. 2013). To date, 3 articles have reported the role of microRNA-141 in NSCLC. However, the results were contradictory (Liu et al. 2012; Mei et al. 2014; Tejero et al. 2014). Mei et al. (2014) and Liu et al. (2012) found that the expression of microRNA-141 in NSCLC tissues were up-regulated, whereas Tejero et al. (2014) showed that the level of microRNA-141 in NSCLC tissues was not significantly different from that in the adjacent non-cancerous lung tissues. In addition, Liu et al. (2012) found that the high expression of microRNA-141 was associated with poor prognosis of NSCLC. However, Tejero et al. (2014) showed that high microRNA-141 expression correlated with shorter overall survival in ADC, but not in SCC. Moreover, the relationship between microRNA-141 level and clinicopathological parameters has been not studied in NSCLC. Therefore, we examined the expression of microRNA-141 in 125 paired NSCLC and adjacent non-cancerous lung tissues, and evaluated its contribution to the NSCLC progression. Furthermore, we studied the relationship between microRNA-141 and the status of EGFR, a mature molecular target for the personalized therapy in NSCLC, including EGFR amplification, EGFR protein expression and EGFR mutations sensitive to tyrosine kinase inhibitor (TKI) treatment.

Materials and Methods

Patients and tissue samples

One hundred twenty-five paired formalin-fixed, paraffin embedded (FFPE) NSCLC tissues and adjacent non-cancerous lung tissues were obtained from the patients who underwent primary surgical resection of NSCLC between January, 2012 and February, 2014 at the First Affiliated Hospital of Guangxi Medical University in China. The Ethical Committee of First Affiliated Hospital, Guangxi Medical University, China approved the current research, and informed consent was obtained from all patients involved. All samples were reviewed and diagnosed by two independent pathologists. Table 1 showed the main clinical characteristics for all 125 patients. Median age was 61 years (range, 23-90) and 60% cases were males. One hundred and one (80.8%) patients were ADC, 23 (18.4%) SCC and 1 (0.8%) LCC. Fifty-four (43.2%) patients had TNM stage I or II disease. Median follow-up was 13 months (range, 1-50.6) for 57 patients. The EGFR status was detected as previously reported (Chen et al. 2011, 2012, 2013b).

RNA preparation, reverse transcription and quantitative real-time PCR

Total RNAs were extracted from FFPE cancer and non-cancerous tissues by using miRNeasy FFPE Kit (QIAGEN, KJ Venlo, Netherlands) under the manufacturer protocol. Reverse transcription (RT) and qPCR kits were applied to evaluate expression of microRNA-141 as described previously (Chen et al. 2011, 2012, 2013b; Rong et al. 2014). Previously, we found that the combination of microRNA-191 and microRNA-103 was the most stable housekeeping microRNA by using NormFinder and geNorm. This combination was used in the current study for the detection of microRNA-141 expression. The primers for microRNA-141, microRNA-191 and microRNA-103 were included in TaqMan® MicroRNA Assays (4427975, Applied Biosystems, Life Technologies Grand Island, NY, 14072, USA). Sequence of microRNA and references used in the paper are: microRNA-141 (Applied Biosystems Cat. No. 4427975-000463): UAACACUGUCUGGUAAAGAUGG; microRNA-191 (Applied Biosystems Cat. No. 4427975-000490): CAACGGAAUCCCAAAAGCAGCU; microRNA-103 (Applied Biosystems Cat. No. 4427975-000439): AGCAGCAUUGUACAG

Table 1. The relationship between microRNA-141 and clinicopathological parameters in NSCLC.

Clinicopathological feature		*n*	microRNA-141 relevant expression($2^{-\Delta Cq}$)		
			Mean ± S.D.	*t*	*P*-value
Tissue	NSCLC	125	7.235 ± 7.415	4.724[a]	< 0.001
	Adjacent non-cancerous lung	125	3.678 ± 4.625		
Age (years)	< 60	57	8.067 ± 9.040	1.151	0.252
	≥ 60	68	6.537 ± 5.689		
Gender	Male	75	7.193 ± 8.220	−0.076	0.940
	Female	50	7.296 ± 6.091		
Smoking	No	38	7.751 ± 10.367	0.304	0.762
	Yes	30	7.130 ± 4.706		
Tumor size (cm)	≤ 3	60	5.145 ± 8.654	−3.133	0.002
	> 3	65	9.164 ± 5.442		
Lymph node metastasis	No	56	5.508 ± 4.415	−2.389	0.018
	Yes	69	8.635 ± 8.947		
Vascular invasion	No	90	7.645 ± 8.0423	0.993	0.323
	Yes	35	6.178 ± 5.442		
TNM	I-II	54	5.498 ± 4.052	−2.323	0.022
	III-IV	71	8.555 ± 8.993		
Pathological grading	I	17	6.292 ± 5.110	0.236[b]	0.791
	II	78	7.559 ± 8.528		
	III	30	6.924 ± 5.164		
Histological classification	Adenocarcinoma	101	6.751 ± 7.819	−1.210[b]	0.302
	Squamous cell carcinoma	23	9.138 ± 5.097		
	Large cell carcinoma	1	12.3		
EGFR amplification	No	39	6.769 ± 4.310	−1.187	0.24
	Yes	18	8.401 ± 5.817		
EGFR protein expression	low	40	7.282 ± 4.529	−0.007	0.995
	high	17	7.291 ± 5.668		
EGFR mutation	Wild type	44	7.451 ± 4.716	0.476	0.636
	Mutation[c]	13	6.719 ± 5.416		

[a]Paired *t* student's test was performed.

[b]One-way analysis of variance (ANOVA) test was performed.

[c]EGFR mutation included short in-frame deletions in exon 19 and point mutations that result in a substitution of arginine for leucine at codon 858 (L858R) in exon 21.

Smoking history was collected for only 68 cases and EGFR amplification, protein expression and mutation were detected in 57 cases.

GGCUAUGA. The reverse primers were also used for the reverse transcription with TaqMan® MicroRNA Reverse Transcription Kit (4366596, Applied Biosystems, Life Technologies Grand Island, NY, 14072, USA) in a total volume of 10 μl. Real-time qPCR for microRNA was performed with Applied Biosystems PCR7900. The microRNA-141 expression in FFPE experiment was calculated with the formula $2^{-\Delta cq}$ (Chen et al. 2013b; Dang et al. 2013, 2014).

Statistical analysis

The statistical analyses were performed using SPSS 20.0 software (SPSS, Inc., Munich, Germany). Paired sample *t*-test was used to compare the difference of microRNA-141 expression between NSCLC tissue and adjacent non-cancerous lung tissue. Comparisons between unpaired groups were conducted using the student *t*-test. One-way analysis of variance (ANOVA) test was used to analyze the correlation between the expression levels of microRNA-141 and pathological grading and histological classification. Receiver operating characteristic (ROC) curve was generated to evaluate the power of microRNA-141 to distinguish the NSCLC patients from non-cancerous lung tissue. Survival analysis was estimated by the Kaplan-Meier method, and the log-rank test was used to compare the survival between groups. Cox proportional hazard regression model was applied to analyze the risk factors for NSCLC. A *P*-value less than 0.05, which was calculated by two-tailed test, was considered statistically significant.

Results

Expression of microRNA-141 in NSCLC tissues

The microRNA-141 expression levels were significantly higher in NSCLC tissues compared with adjacent non-cancerous lung tissues ($P < 0.001$, Fig. 1A). For sub-

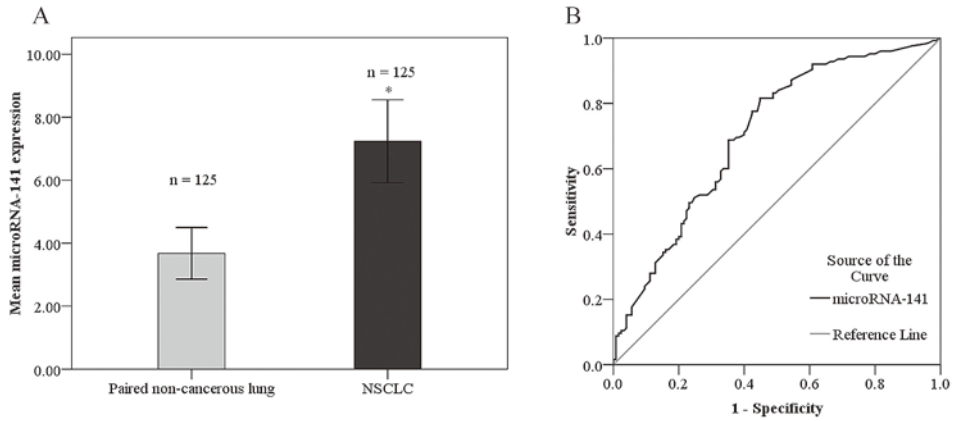

Fig. 1. MicroRNA-141 expression and its diagnostic value in NSCLC.
Quantitative real-time RT-PCR was performed to detect the expression of microRNA-141 in non-small cell lung cancer (NSCLC) and the paired adjacent non-cancerous lung tissue (A). *$P < 0.001$. ROC curve of microRNA-141 level in NSCLC (B). The area under curve (AUC) of microRNA-141 was 0.707 (95% CI 0.643~0.771).

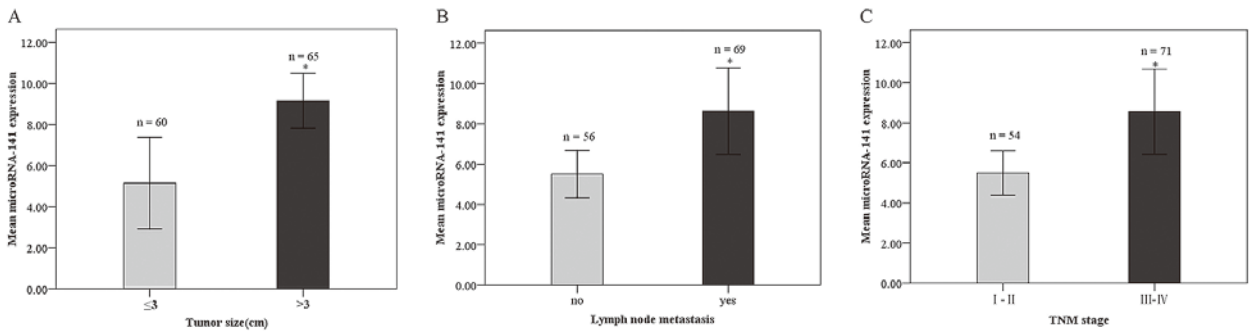

Fig. 2. Relationship between microRNA-141 expression and some clinicopathological features in NSCLC.
A: lymphatic metastasis; B: tumor size; C: clinical TNM stages. *$P < 0.05$.

group of histological classification, microRNA-141 expression levels were both elevated in SCC or ADC, compared with corresponding lung tissues ($P < 0.01$). Furthermore, ROC curve was performed to identify the diagnostic value of microRNA-141 level in NSCLC. The area under curve (AUC) of microRNA-141 was 0.707 (95% CI 0.643~0.771, $P < 0.001$). The cut-off value for microRNA-141 was 3.32. The sensitivity and specificity were 64.8% and 64.8%, respectively (Fig. 1B). Meanwhile, ROC curve was performed to identify the diagnostic value of microRNA-141 level in SCC. The AUC of microRNA-141 was 0.750 (95% CI 0.662~0.837, $P < 0.001$). The cut-off value for microRNA-141 was 5.54. The sensitivity and specificity were 73.9% and 69.2%, respectively. For ADC, the AUC of microRNA-141 was 0.622 (95% CI 0.553~0.691, $P = 0.001$). The cut-off value for microRNA-141 was 3.32. The sensitivity and specificity were 62.4% and 58.4%, respectively.

Relationship between microRNA-141 expression and clinicopathological parameter in NSCLC

Higher expression of microRNA-141 was found in the groups of larger tumor size, lymph node metastasis and TNM stage III-IV than in the corresponding groups (all $P < 0.05$, Table 1, Fig. 2A-C). Moreover, according to Spearman correlation test, microRNA-141 expression level was found to correlate with tumor size ($r = 0.272$, $P = 0.002$), lymph node metastasis ($r = 0.211$, $P = 0.018$) and TNM stage ($r = 0.205$, $P = 0.022$). However, no significant relationship was detected between microRNA-141 level and other clinicopathological parameters, such as age, gender, smoke, vascular invasion, pathological grading, histological classification, EGFR amplification, EGFR protein expression and EGFR mutation status (all $P > 0.05$, Table 1). For subgroup analysis, the relationships between microRNA-141 and clinicopathological parameter in SCC or ADC are shown in Tables 2 and 3, respectively. According to Spearman correlation test, microRNA-141 expression level in SCC was found to correlate with tumor size ($r = 0.490$, $P = 0.018$). While in ADC, microRNA-141 level was positive association with some clinicopathological parameters, such as tumor size ($r = 0.222$, $P = 0.026$), lymph node metastasis ($r = 0.242$, $P = 0.015$) and TNM stage ($r = 0.210$, $P = 0.035$).

Correlation between microRNA-141 expression and prognosis of ADC patients

As the survival data was limited, we could only

Table 2. The relationship between microRNA-141 and clinicopathological parameters in lung squamous cell carcinoma.

Clinicopathological feature		*n*	microRNA-141 relevant expression($2^{-\Delta Cq}$)		
			Mean ± S.D.	*t*	*P*-value
Tissue	Squamous cell carcinoma	23	9.138 ± 5.097	7.611[a]	< 0.001
	Adjacent non-cancerous lung	23	0.895 ± 1.001		
Age (years)	< 60	15	10.277 ± 4.966	1.051	0.146
	≥ 60	8	7.004 ± 4.933		
Gender	Male	18	9.262 ± 5.036	0.216	0.831
	Female	5	8.692 ± 5.895		
Smoke	No	12	8.637 ± 5.445	−0.484	0.633
	Yes	11	9.686 ± 4.884		
Tumor size (cm)	≤ 3	7	5.443 ± 5.114	−2.578	0.018
	> 3	16	10.756 ± 4.298		
Lymph node metastasis	No	11	9.026 ± 5.127	−0.99	0.922
	Yes	12	9.241 ± 5.294		
Vascular invasion	No	20	9.949 ± 4.948	2.121	0.046
	Yes	3	3.733 ± 1.665		
TNM	I-II	10	8.175 ± 4.486	−0.788	0.439
	III-IV	13	9.879 ± 5.582		
Pathological grading	I	0	–	0.504[b]	0.486
	II	16	8.634 ± 5.305		
	III	7	10.291 ± 4.758		
EGFR amplification	No	17	8.488 ± 4.575	−1.031	0.314
	Yes	6	10.980 ± 6.467		
EGFR protein expression	low	18	8.517 ± 4.715	−1.115	0.277
	high	5	7.291 ± 5.668		
EGFR mutation	Wild type	23	9.138 ± 5.097	–	–
	Mutation[c]	0			

[a]Paired *t* student's test was performed.

[b]One-way analysis of variance (ANOVA) test was performed.

[c]EGFR mutation included short in-frame deletions in exon 19 and point mutations that result in a substitution of arginine for leucine at codon 858 (L858R) in exon 21.

explore the relationship between microRNA-141 expression and prognosis of ADC. We employed Kaplan-Meier method, log-rank test and univariate Cox proportional hazard regression model to analyze the survival of ADC patients with the expression of microRNA-141 and other clinicopathological features. Among the 57 patients followed up, 26 had higher microRNA-141 levels, compared to the mean level of 5.5464, and 31 had lower microRNA-141 levels. The survival of high microRNA-141 expression group was 12.1 ± 2.6 months, significantly shorter than that of the low expression group (24.2 ± 3.6 months, $P = 0.037$, Fig. 3) as shown with Kaplan-Meier method. No other clinicopathological parameters showed significant impact on survival of ADC. Furthermore, univariate analysis of overall survival revealed that the relative level of microRNA-141 expression ($P = 0.041$) was prognostic indicator for ADC (Table 4).

Discussion

MicroRNA-141 expression has been reported to be down-regulated or up-regulated in different malignancies, which raises a controversial issue for the role of microRNA-141. Several research groups have reported the expression level of microRNA-141 in NSCLC. Tejero et al. (2014) found no significant difference in microRNA-141 expression between NSCLC (SCC $n = 70$ and ADC $n = 73$) and their adjacent non-cancerous lung tissues in a Spanish population. Inversely, in the present study, microRNA-141 level was 1.97-fold higher in NSCLC (all $n = 125$: SCC $n = 23$, ADC $n = 101$, and LCC $n = 1$) than in the paired adjacent non-cancerous lung tissues. These results were concordant with the studies with small size of NSCLC patients (all $n = 70$: SCC $n = 36$ and ADC $n = 34$) (Liu et al. 2012) and NSCLC ($n = 12$) (Mei et al. 2014). The number of SCC may be a factor for the contradicting results. Since the studies of Liu et al. (2012), Mei et al. (2014) and ours were all based on Chinese NSCLC patients, it could be possible that microRNA-141 expression may be related to the race, e.g., microRNA-141 may play a different role in the

Table 3. The relationship between microRNA-141 and clinicopathological parameters in lung adenocarcinoma.

Clinicopathological feature		*n*	microRNA-141 relevant expression($2^{-\Delta Cq}$)		
			Mean ± S.D.	*t*	*P*-value
Tissue	Adenocarcinoma	101	6.751 ± 7.819	2.704[a]	0.007
	Adjacent non-cancerous lung	101	4.343 ± 4.897		
Age (years)	< 60	41	7.155 ± 10.132	0.428	0.67
	≥ 60	60	5.061 ± 5.347		
Gender	Male	56	6.437 ± 8.978	−0.448	0.655
	Female	45	7.141 ± 6.157		
Smoke	No	26	7.342 ± 12.060	0.699	0.488
	Yes	18	5.281 ± 3.785		
Tumor size (cm)	≤ 3	53	5.105 ± 9.052	0.714	0.026
	> 3	48	8.568 ± 5.744		
Lymph node metastasis	No	45	4.648 ± 3.813	−2.484	0.015
	Yes	56	8.440 ± 9.644		
Vascular invasion	No	70	6.987 ± 8.643	0.455	0.650
	Yes	31	6.127 ± 5.616		
TNM	I-II	44	4.890 ± 3.738	−2.139	0.035
	III-IV	57	8.187 ± 9.676		
Pathological grading	I	17	6.292 ± 5.110	0.262[b]	0.770
	II	61	7.200 ± 9.246		
	III	23	6.751 ± 7.819		
EGFR amplification	No	21	5.114 ± 3.411	−1.178	0.256
	Yes	12	7.112 ± 5.277		
EGFR protein expression	low	22	6.270 ± 4.209	0.824	0.416
	high	11	4.980 ± 4.303		
EGFR mutation	Wild type	20	5.269 ± 3.252	−0.869	0.397
	Mutation[c]	13	6.719 ± 5.416		

[a]Paired *t* student's test was performed.

[b]One-way analysis of variance (ANOVA) test was performed.

[c]EGFR mutation included short in-frame deletions in exon 19 and point mutations that result in a substitution of arginine for leucine at codon 858 (L858R) in exon 21.

Smoking history was collected for only 44 cases and EGFR amplification, protein expression and mutation were detected in 33 cases.

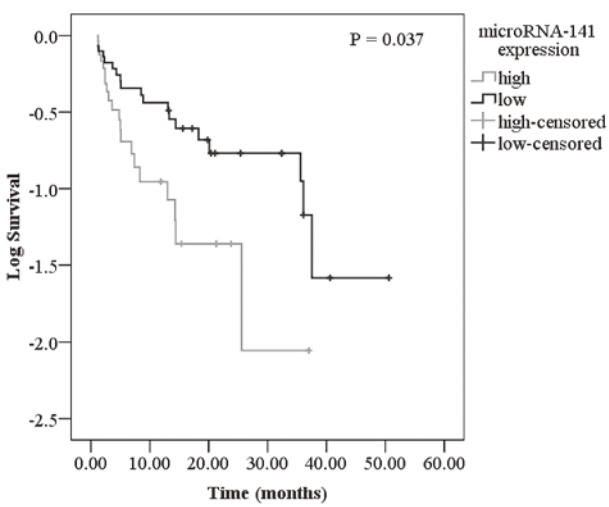

Fig. 3. Kaplan-Meier survival curves for NSCLC patients. NSCLC patients with high microRNA-141 expression had a significantly poorer prognosis than those with low expression ($P = 0.037$).

NSCLC of yellow race and white race. However, this hypothesis needs to be verified and thoroughly investigated. Besides, for the first time, we employed ROC curve to analyze the diagnostic value of microRNA-141 level in NSCLC patients. As a result, ROC demonstrated that microRNA-141 had a moderate diagnostic value for NSCLC with the AUC of 0.707. Meanwhile, microRNA-141 level in SCC had higher diagnosis value than that in ADC. Together with literatures, the current finding strongly indicates the possible role of microRNA-141 as an oncogenic microRNA in NSCLC and the potential characteristic of microRNA-141 in the carcinogenesis of NSCLC.

Interestingly, we found that the relative expression of microRNA-141 was lower in the SCC-adjacent tissues than that in the ADC-adjacent tissues. This difference could not be explained by the histological morphology, since no obvious distinction of the adjacent tissues between SCC and ADC was found, except that in some cases of SCC-adjacent tissues, squamous metaplasia could be observed. We

Table 4. Univariate Analysis of clinicopathological factors for overall survival in adenocarcinoma.

	Subset	HR	95% CI	*P*-value
Age (years)	< 60/≥ 60	0.956	0.470-1.945	0.901
Gender	Male/Female	0.779	0.404-1.502	0.456
TNM	I-II/III-IV	1.955	0.921-4.153	0.081
Tumor size (cm)	≤ 3/> 3	1.396	0.725-2.691	0.318
Lymph node metastasis	No/Yes	1.351	0.681-2.683	0.389
Vascular invasion	No/Yes	1.059	0.562-1.995	0.860
Pathological grading	I/II-III	0.978	0.5-1.914	0.948
Pathological grading	I-II/III	0.785	0.306-2.017	0.615
MicroRNA-141	Low/High	1.965	1.027-3.760	0.041

hypothesize that the altered unclarified mechanisms of microRNA-141 expression in the tumorigenesis of SCC and ADC lead to the variation of the expression level in SCC- and ADC-adjacent tissues. Further work should include absolutely normal lung tissues as controls, and *in vitro* and *in vivo* experiments would also be required to figure out this issue.

MicroRNAs are proved to be more stable and reproducible than mRNA (Ramshankar and Krishnamurthy 2013). Thus, circulating microRNAs might be considered as blood-based biomarker in cancer diagnosis and prognosis (Qu et al. 2011). However, Liu et al. (2012) failed to test the aberrant microRNA-141 expression in serum of NSCLC patients. Quantitative RT-PCR results showed that serum level of microRNA-141 in NSCLC patients was not obviously different from normal volunteers, which was inconsistent with the overexpression in NSCLC tissues. Thus, the expression profiles of microRNA-141 were variable in NSCLC tissues and sera, suggesting that serum microRNA-141 might not truly reflect the level of tumor microRNAs. We also compared some microRNA profiles in the NSCLC tissues and their paired serum samples, and found the similar conflicting expression patterns (data not shown). The relationship between tissue microRNAs and serum microRNAs is uncertain. Cancer heterogeneity of primary tumor cells and circulating tumor cells could be one of the potential explanations for this discrepancy. Thus, the clinical significance of serum microRNA-141 remains to be determined.

Regarding the relationship between microRNA-141 expression and clinicopathological parameters, Liu et al. (2012) determined no association between microRNA-141 level and any clinicopathological feature. In the present study, microRNA-141 expression with larger tumor was found to be significantly higher than that with smaller ones. Meanwhile, microRNA-141 expression was significantly upregulated in lymph node metastatic group compared to that in the non-metastatic group. Interestingly, when NSCLC patients were divided into SCC, ADC and LCC, we found that microRNA-141 expression level in SCC was associated with tumor size. Additionally, microRNA-141 expression was correlated with the clinical TNM stage. The status of tumor size and lymph node metastasis generally reflects tumor growth, invasion and metastasis and the disease deterioration. Thus, the result in current study reveals an obvious relationship between the high expression level of microRNA-141 and the progression of NSCLC. Furthermore, patients with high microRNA-141 level tended to have shorter survival; namely, microRNA-141 was an independent prognostic indicator to predict the prognosis of NSCLC, which was accordant with Liu et al. (2012) and Tejero et al. (2014). Hence it may be valuable to examine microRNA-141 expression for the prediction of deterioration and prognosis of NSCLC.

Treatment strategies for NSCLC continue to evolve, most of which have produced positive trial results for EGFR TKIs in the first-line setting in molecularly targeted populations (Zheng et al. 2013). Thus, we were also interested in the relationship between microRNA-141 expression and the EGFR status. However, no correlation was found between microRNA-141 level and all EGFR status, including EGFR amplification, EGFR protein expression and EGFR mutations in exon 19 and exon 21.

Concerning the mechanism of microRNA-141 being upregulated in advanced stage of NSCLC, different target genes and pathways involved could play vital roles. Diverse target genes and related signaling of microRNA-141 have been demonstrated in NSCLC. Tejero et al. (2014) found that overexpression of microRNA-141 reduced KLF6 protein levels and produced an increase of secretion of VEGFA *in vitro* and microRNA-141 was associated with higher blood micro vessel density in patient tumor samples. Mei et al. (2014) reported that microRNA-141 directly targets PH domain leucine-rich-repeats protein phosphatase 1 (PHLPP1) and PHLPP2, which enhance the proliferation of NSCLC cells by promoting cell cycle progression. The in-depth mechanism of microRNA-141 needs further investigation.

In conclusion, microRNA-141 level increases in NSCLC tissues. The high expression level of microRNA-141 was closely related to the poor prognosis of NSCLC. Therefore, microRNA-141 may be a new biomarker for molecular diagnosis, risk evaluation and prognosis prediction of NSCLC.

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Conflict of Interest

The authors declare no conflict of interest.

References

Asai, N., Ohkuni, Y., Kaneko, N., Yamaguchi, E. & Kubo, A. (2014) Relapsed small cell lung cancer: treatment options and latest developments. *Ther. Adv. Med. Oncol.*, **6**, 69-82.

Brothers, J.F., Hijazi, K., Mascaux, C., El-Zein, R.A., Spitz, M.R. & Spira, A. (2013) Bridging the clinical gaps: genetic, epigenetic and transcriptomic biomarkers for the early detection of lung cancer in the post-National Lung Screening Trial era. *BMC Med.*, **11**, 168.

Chen, B., Huang, T., Jiang, J., Lv, L., Li, H. & Xia, S. (2014a) miR-141 suppresses proliferation and motility of gastric cancer cells by targeting HDGF. *Mol. Cell. Biochem.*, **388**, 211-218.

Chen, G., Kronenberger, P., Teugels, E. & De Greve, J. (2011) Influence of RT-qPCR primer position on EGFR interference efficacy in lung cancer cells. *Biol. Proced. Online*, **13**, 1.

Chen, G., Kronenberger, P., Teugels, E., Umelo, I.A. & De Greve, J. (2012) Targeting the epidermal growth factor receptor in non-small cell lung cancer cells: the effect of combining RNA interference with tyrosine kinase inhibitors or cetuximab. *BMC Med.*, **10**, 28.

Chen, G., Kronenberger, P., Teugels, E., Umelo, I.A. & De Greve, J. (2013a) Effect of siRNAs targeting the EGFR T790M mutation in a non-small cell lung cancer cell line resistant to EGFR tyrosine kinase inhibitors and combination with various agents. *Biochem. Biophys. Res. Commun.*, **431**, 623-629.

Chen, G., Umelo, I.A., Lv, S., Teugels, E., Fostier, K., Kronenberger, P., Dewaele, A., Sadones, J., Geers, C. & De Greve, J. (2013b) miR-146a inhibits cell growth, cell migration and induces apoptosis in non-small cell lung cancer cells. *PLoS One*, **8**, e60317.

Chen, X., Wang, X., Ruan, A., Han, W., Zhao, Y., Lu, X., Xiao, P., Shi, H., Wang, R., Chen, L., Chen, S., Du, Q., Yang, H. & Zhang, X. (2014b) miR-141 is a key regulator of renal cell carcinoma proliferation and metastasis by controlling EphA2 expression. *Clin. Cancer Res.*, **20**, 2617-2630.

Dang, Y., Luo, D., Rong, M. & Chen, G. (2013) Underexpression of miR-34a in hepatocellular carcinoma and its contribution towards enhancement of proliferating inhibitory effects of agents targeting c-MET. *PLoS One*, **8**, e61054.

Dang, Y.W., Zeng, J., He, R.Q., Rong, M.H., Luo, D.Z. & Chen, G. (2014) Effects of miR-152 on cell growth inhibition, motility suppression and apoptosis induction in hepatocellular carcinoma cells. *Asian Pac. J. Cancer Prev.*, **15**, 4969-4976.

De Mello, R.A., Araujo, A., Hespanhol, V. & Reis, R.M. (2013) Loci identified through genome-wide association studies and lung cancer risk: is there anything more? *Sao Paulo Med. J.*, **131**, 135-136.

DeSantis, C.E., Lin, C.C., Mariotto, A.B., Siegel, R.L., Stein, K.D., Kramer, J.L., Alteri, R., Robbins, A.S. & Jemal, A. (2014) Cancer treatment and survivorship statistics, 2014. *CA Cancer J. Clin.*, **64**, 252-271.

Di Leva, G., Garofalo, M. & Croce, C.M. (2014) MicroRNAs in cancer. *Annu. Rev. Pathol.*, **9**, 287-314.

Gazala, S., Pelletier, J.S., Storie, D., Johnson, J.A., Kutsogiannis, D.J. & Bedard, E.L. (2013) A systematic review and meta-analysis to assess patient-reported outcomes after lung cancer surgery. *ScientificWorldJournal*, **2013**, 789625.

Gridelli, C., de Marinis, F., Cappuzzo, F., Di Maio, M., Hirsch, F.R., Mok, T., Morgillo, F., Rosell, R., Spigel, D.R., Yang, J.C. & Ciardiello, F. (2014) Treatment of advanced non-small-cell lung cancer with epidermal growth factor receptor (EGFR) mutation or ALK gene rearrangement: results of an international expert panel meeting of the Italian Association of Thoracic Oncology. *Clin. Lung Cancer*, **15**, 173-181.

Guz, M., Rivero-Muller, A., Okon, E., Stenzel-Bembenek, A., Polberg, K., Slomka, M. & Stepulak, A. (2014) MicroRNAs-role in lung cancer. *Dis. Markers*, **2014**, 218169.

Hu, Z., Chen, J., Tian, T., Zhou, X., Gu, H., Xu, L., Zeng, Y., Miao, R., Jin, G., Ma, H., Chen, Y. & Shen, H. (2008) Genetic variants of miRNA sequences and non-small cell lung cancer survival. *J. Clin. Invest.*, **118**, 2600-2608.

Iuliano, R., Vismara, M.F., Dattilo, V., Trapasso, F., Baudi, F. & Perrotti, N. (2013) The role of microRNAs in cancer susceptibility. *Biomed Res. Int.*, **2013**, 591931.

Jemal, A., Bray, F., Center, M.M., Ferlay, J., Ward, E. & Forman, D. (2011) Global cancer statistics. *CA Cancer J. Clin.*, **61**, 69-90.

Kang, S.M. & Lee, H.J. (2014) MicroRNAs in human lung cancer. *Exp. Biol. Med.* (*Maywood*), **239**, 1505-1513.

Kuwabara, K., Matsuda, S., Fushimi, K., Anan, M., Ishikawa, K.B., Horiguchi, H., Hayashida, K. & Fujimori, K. (2009) Differences in practice patterns and costs between small cell and non-small cell lung cancer patients in Japan. *Tohoku J. Exp. Med.*, **217**, 29-35.

Lauro, S., Onesti, C.E., Righini, R. & Marchetti, P. (2014) The use of bevacizumab in non-small cell lung cancer: an update. *Anticancer Res.*, **34**, 1537-1545.

Liu, H.Y. & Chen, J. (2011) Polymorphisms in miRNA binding site: new insight into small cell lung cancer susceptibility. *Acta Pharmacol. Sin.*, **32**, 1191-1192.

Liu, X.G., Zhu, W.Y., Huang, Y.Y., Ma, L.N., Zhou, S.Q., Wang, Y.K., Zeng, F., Zhou, J.H. & Zhang, Y.K. (2012) High expression of serum miR-21 and tumor miR-200c associated with poor prognosis in patients with lung cancer. *Med. Oncol.*, **29**, 618-626.

Liu, Y., Ding, Y., Huang, J., Wang, S., Ni, W., Guan, J., Li, Q., Zhang, Y., Ding, Y., Chen, B. & Chen, L. (2014) MiR-141 suppresses the migration and invasion of HCC cells by targeting Tiam1. *PLoS One*, **9**, e88393.

Mei, Z., He, Y., Feng, J., Shi, J., Du, Y., Qian, L., Huang, Q. & Jie, Z. (2014) MicroRNA-141 promotes the proliferation of non-small cell lung cancer cells by regulating expression of PHLPP1 and PHLPP2. *FEBS Lett.*, **588**, 3055-3061.

Oom, A.L., Humphries, B.A. & Yang, C. (2014) MicroRNAs: novel players in cancer diagnosis and therapies. *Biomed. Res. Int.*, **2014**, 959461.

Qu, H., Xu, W., Huang, Y. & Yang, S. (2011) Circulating miRNAs: promising biomarkers of human cancer. *Asian Pac. J. Cancer Prev.*, **12**, 1117-1125.

Ramshankar, V. & Krishnamurthy, A. (2013) Lung cancer detection by screening - presenting circulating miRNAs as a promising next generation biomarker breakthrough. *Asian Pac. J. Cancer Prev.*, **14**, 2167-2172.

Ritchie, W. & Rasko, J.E. (2014) Refining microRNA target predictions: sorting the wheat from the chaff. *Biochem. Biophys. Res. Commun.*, **445**, 780-784.

Rong, M., He, R., Dang, Y. & Chen, G. (2014) Expression and clinicopathological significance of miR-146a in hepatocellular carcinoma tissues. *Ups. J. Med. Sci.*, **119**, 19-24.

Siegel, R., Ma, J., Zou, Z. & Jemal, A. (2014) Cancer statistics, 2014. *CA Cancer J. Clin.*, **64**, 9-29.

Skrzypski, M., Czapiewski, P., Goryca, K., Jassem, E., Wyrwicz,

L., Pawlowski, R., Rzyman, W., Biernat, W. & Jassem, J. (2014) Prognostic value of microRNA expression in operable non-small cell lung cancer patients. *Br. J. Cancer*, **110**, 991-1000.

Tejero, R., Navarro, A., Campayo, M., Vinolas, N., Marrades, R.M., Cordeiro, A., Ruiz-Martinez, M., Santasusagna, S., Molins, L., Ramirez, J. & Monzo, M. (2014) miR-141 and miR-200c as markers of overall survival in early stage non-small cell lung cancer adenocarcinoma. *PLoS One*, **9**, e101899.

Usui, S., Minami, Y., Shiozawa, T., Iyama, S., Satomi, K., Sakashita, S., Sato, Y. & Noguchi, M. (2013) Differences in the prognostic implications of vascular invasion between lung adenocarcinoma and squamous cell carcinoma. *Lung Cancer*, **82**, 407-412.

Vageli, D.P., Zaravinos, A., Daniil, Z., Dahabreh, J., Doukas, S.G., Spandidos, D.A., Gourgoulianis, K.I. & Koukoulis, G.K. (2012) hMSH2 and hMLH1 gene expression patterns differ between lung adenocarcinoma and squamous cell carcinoma: correlation with patient survival and response to adjuvant chemotherapy treatment. *Int. J. Biol. Markers*, **27**, e400-404.

Wang, Y.C., Sung, W.W., Wang, L., Cheng, Y.W., Chen, C.Y., Wu, T.C., Shieh, S.H. & Lee, H. (2013) Different impact of IL10 haplotype on prognosis in lung squamous cell carcinoma and adenocarcinoma. *Anticancer Res.*, **33**, 2729-2735.

Wu, S.M., Ai, H.W., Zhang, D.Y., Han, X.Q., Pan, Q., Luo, F.L. & Zhang, X.L. (2014) miR-141 targets ZEB2 to suppress HCC progression. *Tumour Biol.*, **35**, 9993-9997.

Xia, H., Sun, S., Wang, B., Wang, T., Liang, C., Li, G., Huang, C., Qi, D. & Chu, X. (2014a) miR-143 inhibits NSCLC cell growth and metastasis by targeting Limk1. *Int. J. Mol. Sci.*, **15**, 11973-11983.

Xia, Y., Wu, Y., Liu, B., Wang, P. & Chen, Y. (2014b) Downregulation of miR-638 promotes invasion and proliferation by regulating SOX2 and induces EMT in NSCLC. *FEBS Lett.*, **588**, 2238-2245.

Zhao, G., Wang, B., Liu, Y., Zhang, J.G., Deng, S.C., Qin, Q., Tian, K., Li, X., Zhu, S., Niu, Y., Gong, Q. & Wang, C.Y. (2013) miRNA-141, downregulated in pancreatic cancer, inhibits cell proliferation and invasion by directly targeting MAP4K4. *Mol. Cancer Ther.*, **12**, 2569-2580.

Zheng, D.J., Yu, G.H., Gao, J.F. & Gu, J.D. (2013) Concomitant EGFR inhibitors combined with radiation for treatment of non-small cell lung carcinoma. *Asian Pac. J. Cancer Prev.*, **14**, 4485-4494.

Zhou, X., Xia, Y., Su, J. & Zhang, G. (2014) Down-regulation of miR-141 induced by helicobacter pylori promotes the invasion of gastric cancer by targeting STAT4. *Cell. Physiol. Biochem.*, **33**, 1003-1012.