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Potential use of microRNA-200c as a prognostic marker in non-small cell lung cancer

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Abstract. MicroRNAs (miRNAs/miRs) are a class of small, highly conserved non-coding RNAs that can serve either oncogenic or tumor-suppressive roles in a wide variety of tumors. miR-200c is a member of the miR-200 family whose specific role in non-small cell lung cancer (NSCLC) has not yet been elucidated. The purpose of the present study was to detect the expression level of miR-200c in NSCLC, and to analyze its association with clinicopathological factors and patient prognosis. The present study determined the expression levels of miR-200c in 110 tumor samples collected from patients diagnosed with NSCLC who underwent complete tumor resection with regional lymph node dissection, as assessed by reverse transcription-quantitative polymerase chain reaction. The association between the expression level of miR-200c and clinicopathological features and patient prognosis was also analyzed. The results showed that miR-200c overexpression was detected in 66 of the 110 cases and was significantly associated with positive lymph node metastasis (P<0.001). Univariate survival analysis demonstrated that high miR-200c expression, positive lymph node metastasis and advanced Tumor-Node-Metastasis (TNM) classification stage significantly predicted decreased 5-year disease-free survival rates (all P<0.05) and poor 5-year overall survival rates (all P<0.01), respectively. The results of multivariate Cox regression analysis showed that TNM stage and miR-200c expression retained its significance as an independent prognostic factor for unfavorable 5-year disease-free survival rates (P<0.05) and poor 5-year overall survival rates (P<0.01). The present findings suggest that miR-200c overexpression is significantly associated with poor survival rates in NSCLC and that miR-200c could play an oncogenic role. miR-200c may have clinical potential as a promising prognostic predictor for patients with NSCLC.

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Introduction

Lung cancer is the leading cause of cancer-associated mortality worldwide, with ~1.35 million new cases every year worldwide (1). Between 75 and 80% of lung cancer cases are non-small cell lung cancer (NSCLC), which has an overall 5-year survival rate of only 10% (2). Despite novel methods targeting early diagnosis and recent advancements in treatments, the prognosis and survival rate of NSCLC patients remains relatively poor. Between 40 and 50% of patients will eventually succumb to relapse or metastatic disease after curative resection (3). However, few reliable prognostic biomarkers are available in clinical practice, particularly in patients who have undergone curative surgical resection (4). Therefore, there is a requirement to identify novel prognostic biomarkers that could aid prognosis prediction and optimize the treatment of NSCLC patients.

MicroRNAs (miRNAs/miRs) are a class of small (19-24 nucleotides in length), highly conserved non-coding RNAs that bind to the 3'-untranslated regions of target mRNAs and suppress their translation to proteins (5). It has become increasingly evident that different miRNAs can play either oncogenic or tumor-suppressive roles in a wide variety of pathways, depending on the target genes or the cellular context (6,7). miR-200c is a member of the miR-200 family, which consists of five members (miR-200a, miR-200b and miR-429 comprise cluster 1, which is located on chromosome 1p36; and miR-200c and miR-141 comprise cluster 2, which is located on chromosome 12p13) (8). Recent investigations have shown that members of the miR-200 family could promote or repress different cancer types via various pathways (9,10). However, studies investigating the association between the expression level of miR-200c and the clinical outcome in resected NSCLC patients are few in number and have returned contradictory results (8,11,12). The specific role of miR-200c in NSCLC has, therefore, not yet been elucidated.

In the present study, the expression of miR-200c was examined in 110 clinical NSCLC samples and the association between miR-200c expression and variable clinicopathological features and patient prognosis was analyzed. The present study demonstrated that high miR-200c expression levels were associated with poor disease-free and overall survival rates in NSCLC patients after surgery, and that its presence was an independent prognostic factor.

Patients and methods

Patients. A total of 110 tumor samples were collected from patients (65 male, 45 female; mean age, 60.5 years; age range, 41-78, years) who had been pathologically diagnosed with primary NSCLC and who underwent complete tumor resection (lobectomy or pneumonectomy) with regional lymph node dissection at the Department of Thoracic Surgery, Qilu Hospital (Jinan, China) between January and December 2008.

The study was approved by the Institutional Review Board at Qilu Hospital and written informed consent was obtained from all the patients involved in the study. No patients had undergone preoperative radiotherapy or chemotherapy. The postsurgical histological type and grade of cancer cell differentiation was determined by the World Health Organization classification system (revised in 2004), and the pathological Tumor-Node-Metastasis (TNM) classification stage was determined by the 2009 staging system of the Union for International Cancer Control. The complete follow-up data (until December 2015, loss or mortality) were included. The clinicopathological characteristics of these 110 patients are summarized in Table I.

Nucleic acid isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA was extracted from fresh-frozen tissues stored at -80˚C. Reverse transcription was performed with reverse transcription with a Revertaid H Minus First Strand cDNA Synthesis kit (Tiangen Biotech Co., Ltd., Beijing, China) with miRNA-specific primers, using 1 μg of total RNA in a 20 μl reverse transcriptase reaction mixture. miRNA levels were evaluating using SYBR Green PCR Master Mix (Tiangen) for miR-200c and U6 RNA with stem-loop RT-PCR, as described previously (13), following the manufacturer's protocol and using a 20-μl reaction mixture. The reactions were performed on an ABI 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Thermocycling conditions were: 95˚C for 10 min followed by 40 cycles of 95˚C for 15 sec and 62˚C for 1 min. Expression of the U6 small nuclear RNA was used as an internal control to normalize all results. The total RNA of the normal sample was used as a normal control. Data were analyzed using Sequence Detection Software 1.4 (Applied Biosystems; Thermo Fisher Scientific, Inc.). The relative quantity of the transcript was calculated using the $2^{-\Delta\Delta Cq}$ method (14). There were six experimental repeats. Customized primers were designed using the Primer Express software (Applied Biosystems; Thermo Fisher Scientific, Inc.). Primer sequences used are listed in Table II.

Statistical analysis. All statistical analyses were performed using SPSS 18.0 (SPSS, Inc., Chicago, IL, USA). The associations between clinical variables and miR-200c expression were analyzed using the Pearson χ^2 test. Survival curves were plotted using the Kaplan-Meier method and assessed with a log-rank test to identify significant differences, with mortality due to lung cancer as the end point. Multivariate Cox regression was used to perform multivariate survival analysis (5-year disease-free survival and 5-year overall survival). P<0.05 was considered to indicate statistical significance.

Table I. Correlation of clinicopathological variables with miR-200c expression in NSCLC.

Clinical variable	n	Low expression, %	High expression, %	P-value
Age, years				0.051
<60	62	30	32	
≥60	48	14	34	
Sex				0.437
Male	65	24	41	
Female	45	20	25	
Smoking history				1.000
Yes	48	19	29	
No	62	25	37	
Histology				0.112
SCC	66	22	44	
Adenocarcinoma	44	22	22	
Differentiation				0.452
Well	28	10	18	
Moderate	52	24	28	
Poor	30	10	20	
Tumor size, cm				0.172
≤3	46	22	24	
>3	64	22	42	
Lymph node metastasis				0.000
N0	56	29	27	
N1/N2	54	15	39	
TNM stage				0.088
I	46	23	23	
II	45	17	28	
III	19	4	15	

NSCLC, non-small cell lung cancer; miR, microRNA; SCC, squamous cell carcinoma; TNM, tumor-node-metastasis classification system.

Results

Correlation of miR-200c expression with clinicopathological factors. miR-200c levels were quantified by performing stem-loop RT-PCR on 110 NSCLC specimens and 43 normal lung tissues. qPCR confirmed that, compared with their corresponding normal tissues, the NSCLC specimens exhibited upregulated miR-200c expression (Table III; Fig. 1).

The association of miR-200c expression with clinicopathological factors was examined using a χ^2 test. Higher miR-200c expression was significantly associated with positive lymph node metastasis (P<0.001); there was no statistical significance in the associations between miR-200c expression and other clinicopathological variables (P>0.05) (Table I).

Univariate survival analysis for 5-year disease-free survival and 5-year overall survival. Of the 110 NSCLC patients

Table II. Sequences of the primers of miR-200c and U6 RNA.

Primer	miR-200c (5'-3')	U6 RNA (5'-3')
RT	CTCGTATCCAGTGCAGGGTCCG AGGTATTCGCACTGGATACGAGCCAAAC	GTGCAGGGTCCGAGGT
Forward	GAGCCGTCTTACCCAGCA	CTCGCTTCGGCAGCACA
Reverse	GTGCAGGGTCCGAGGTAT	GTGCAGGGTCCGAGGT

RT, reverse transcription; miR, microRNA.

Table III. Expression of miR200c in the 110 NSCLC patients and 43 healthy controls.

Group	Subjects, n	miR200c expression, %[a]	P-value
NSCLC patients	110	15.203±0.575	<0.001
Healthy control	43	5.533±0.684	

[a]Data are presented as the mean ± standard deviation. miR, microRNA; NSCLC, non-small cell lung cancer.

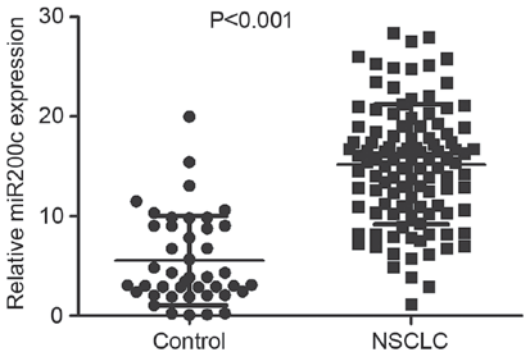

Figure 1. Differential expression of miR-200c between NSCLC patients and the control group was validated by stem-loop reverse transcription-quantitative polymerase chain reaction. miR, microRNA.

examined in this study, tumor relapse developed in 89 (80.9%) within the follow-up period: Local recurrence occurred in 22 patients, distant metastasis in 46 patients, and local recurrence and distant metastasis in 21 patients. Univariate analysis (log-rank test) demonstrated that higher miR-200c expression (15.2 vs. 25.0%, P=0.004; Fig. 2A), positive lymph node metastasis (9.3 vs. 28.6%, P<0.001; Fig. 2B) and advanced TNM stage (0 vs. 15.6 vs. 30.4% for stage III, II, and I, respectively; P=0.011; Fig. 2C) significantly predicted decreased 5-year disease-free survival rates.

Of the 110 NSCLC patients, 74 (67.3%) succumbed to cancer-associated causes within 5 years of surgery, and the 5-year overall survival was 32.7%. Univariate analysis (log-rank test) demonstrated that high miR-200c expression (21.2 vs. 50.0%, P<0.001; Fig. 3A), positive lymph node metastasis (13.0 vs. 51.8%, P<0.001; Fig. 3B) and advanced TNM stage (0 vs. 24.4 vs. 54.3% for stage III, II and I, respectively; P=0.002; Fig. 3C) significantly predicted poor 5-year overall survival rates (Table IV).

Table IV. Univariate survival analysis for disease-free survival and overall survival rates.

Variable	Disease-free survival rate, P-value	Overall survival rate, P-value
Age (≤60 vs. >60 years)	0.406	0.739
Sex (male vs. female)	0.146	0.079
Smoking (yes vs. no)	0.769	0.330
Histology (SCC vs. adenocarcinoma)	0.429	0.844
Differentiation (poor vs. moderate vs. well)	0.911	0.094
Tumor size (≤3 vs. >3 cm)	0.314	0.953
Lymph node metastasis (N0 vs. N1/N2)	0.000	0.000
TNM (stage I vs. stage II/III)	0.011	0.002
miR200c (high vs. low)	0.004	0.000

SCC, squamous cell cancer; TNM, Tumor-Node-Metastasis classification system; miR, microRNA.

Multivariate survival analysis for 5-year disease-free survival and 5-year overall survival. Among all variables, there existed statistical significance for the association between lymph node metastasis, TNM stage and miR-200c expression in univariate survival analysis. Thus, these three variables were assessed using multivariate survival analysis. The results of multivariate Cox regression analysis showed that TNM stage (both P<0.000; Table V) and miR-200c expression (P=0.030 and P=0.006; Table V) retained significance as independent prognostic factors for unfavorable 5-year disease-free survival and poor 5-year overall survival rates, respectively.

Discussion

In the clinic, the prognosis of patients with NSCLC is markedly different, even if they have the same pathological staging (15,16). This difference may be due to the fact that patients are in a different disease stage when they are diagnosed, meaning the current staging system is not sufficient

Table V. Multivariate survival analysis for disease-free survival and overall survival.

	Disease-free survival			Overall survival		
Variable	95% CI	Exp(B)	P-value	95% CI	Exp(B)	P-value
Lymph node metastasis (N0 vs. N1/N2)	0.510-1.626	0.910	0.751	0.453-1.661	0.868	0.668
TNM (stage I vs. stage II and III)	1.857-4.661	2.942	0.000	2.177-5.886	3.580	0.000
miR-200c (high vs. low)	1.049-2.585	1.647	0.030	1.241-3.536	2.095	0.006

CI, confidence interval; TNM, tumor-node-metastasis classification system; miR, microRNA.

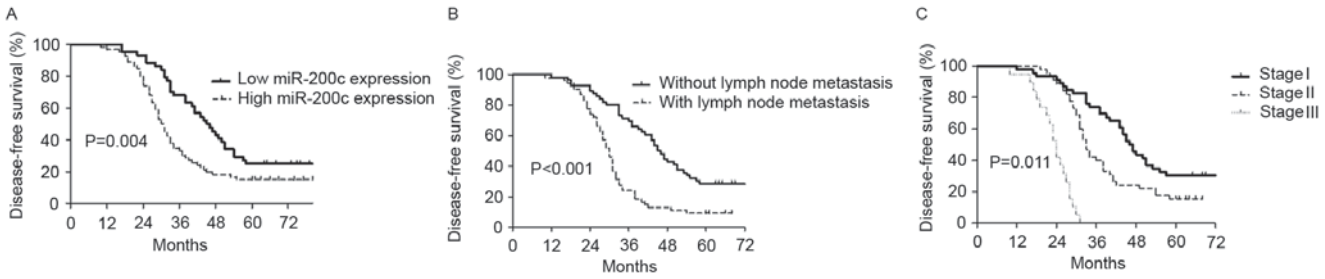

Figure 2. Kaplan-Meier curves of disease-free survival rates, stratified according to (A) miR-200c expression, (B) lymph node metastasis and (C) Tumor-Node-Metastasis stage. miR, microRNA.

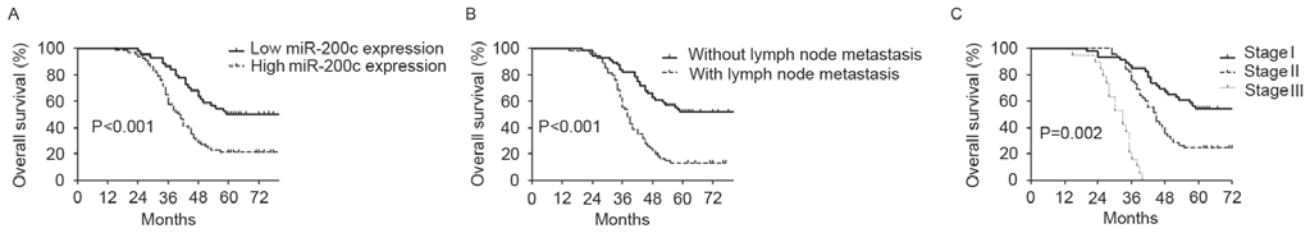

Figure 3. Kaplan-Meier curves of overall survival rates, stratified according to (A) miR-200c expression, (B) lymph node metastasis and (C) TNM stage. miR, microRNA.

to predict prognosis and/or inform a treatment strategy for numerous patients. Therefore, it is necessary to identify and employ novel biomarkers as possible therapeutic targets or prognostic predictors, which will be used as an adjunct to the staging system and contribute to the optimization of treatment for patients with NSCLC. miRNAs as a recent focus in tumor research serve an oncogenic or tumor-suppressive role in different cancer types via various pathways (17-19). miR-200c is a member of the miR-200 family, which is located on chromosome 12p13 and is closely associated with carcinogenesis and disease progression in a wide range of cancer types (20,21). To the best of our knowledge, there have been only 4 clinical studies on miR-200c expression in NSCLC to date, producing contradictory results (i.e. that miR-200c has been reported to have oncogenic or tumor-suppressive functions) (10,12,22,23). The present study aimed to assess miR-200c expression in NSCLC, and to investigate the role of miR-200c in relation to carcinogenesis and the prognosis of NSCLC patients.

The present study demonstrated that miR200c overexpression was common in NSCLC tissues and significantly associated with lymph node metastasis. For 5-year disease-free survival and overall survival rates, Kaplan-Meier analysis showed that patients with lymph node metastasis, advanced TNM stage and high miR-200c expression had a poor prognosis. To ascertain whether the impact of mixed factors was associated with prognosis, Cox regression multivariate analysis was performed and demonstrated that only TNM stage and high miR-200c expression had value as independent prognostic factors. The present results indicate that TNM stage (which is already internationally recognized) and miR200c are useful diagnostic markers that may themselves promote tumor progression in NSCLC and other cancer types.

However, tumor carcinogenesis is a complex process, in which regulation of cell growth and differentiation must be altered (24). Genetic and epigenetic changes can occur at multiple levels, from chromothripsis or the loss or gain of entire chromosomes to a point mutation that alters a single DNA nucleotide, or to the silencing or activation of an miRNA that can alter the expression of up to 500 genes (25,26). To date, the mechanism through which miR-200c can affect the carcinogenic potential of cancer cells remains unknown and requires further elucidation at the molecular level.

The present study indicated that miR-200c is associated with lymph node metastasis, suggesting that miR-200c may be upregulated in the metastatic process. However, miRNA-200c has been shown to restrict the epithelial-mesenchymal transition (EMT) and metastasis through direct targeting of the cell adhesion pathway (27,28), particularly the zinc finger E-box binding homeobox (ZEB)-cadherin 1 axis (29). However, the pleiotropic effect of miR-200c in the metastatic process is contradictory in *in vitro* and *in vivo* studies (30). A previous study demonstrated that the metastatic potential of tumor cells could be increased by the overexpression of miR-200c (31). The overexpression of miR-200c in a xenograft model was found to associate with a higher metastatic potential and increased metastatic colonization (32). The present findings, in which expression of miR-200c was upregulated in tumors and associated with poor prognosis and lymph node metastasis, are consistent with experimental data in ovarian (33), colorectal (34), gastric (35,36) and breast (37) cancer. The mechanism may involve miR-200c overexpression and an increase in metastatic risk by repressing the expression of E-cadherin transcriptional repressors ZEB1 and ZEB2, and the final induction of EMT.

In summary, the present study provides evidence that miR-200c expression in lung cancer tissue may be an effective predictor for monitoring cancer progression and informing on future prognosis, although the underlying mechanism remains unclear. Individual miRNAs can direct different biological processes by regulating the expression of multiple downstream targets, including oncogenes and tumor suppressor genes, which suggests that the contributions of miRNAs to tumorigenesis vary between different tumors. The present results could provide important information for predicting prognosis and tumor progression. However, more well-designed studies with larger sample sizes and a standardized methodology, investigating associated candidate target genes, are required.

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