# Aberrant microRNA-137 promoter methylation is associated with lymph node metastasis and poor clinical outcomes in non-small cell lung cancer

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**Abstract.** MicroRNA-137 (miR-137) functions as a tumor suppressor and is silenced by aberrant promoter methylation. Previous studies have demonstrated that miR-137 is downregulated in lung cancer. The purpose of the present study was to investigate miR-137 promoter methylation and to assess its prognostic value in non-small cell lung cancer (NSCLC). The expression of miR-137 was analyzed inhuman lung cancer A549 and H1299 cells and normal bronchial epithelial BEAS-2B cells, 10 paired formalin-fixed paraffin-embedded lung cancer and normal tissue samples, and 56 archived paraffin-embedded lung cancer tissues. Quantitative methylation-specific polymerase chain reaction analysis was used to assess the miR-137 methylation status. The associations between miR-137 promoter methylation and the clinicopathological features and prognosis of patients with NSCLC (n=56) were analyzed using analysis of variance. miR-137 was markedly downregulated in lung cancer cells and lung cancer tissue specimens compared with expression in BEAS-2B cells and matched adjacent normal lung tissues. A significant negative correlation between miR-137 expression and miR-137 promoter methylation was observed in human lung cancer tissues (r=-0.343; P=0.01). Smoking, lymph node metastasis and advanced clinical stage were associated with significantly lower expression of miR-137 in variance analysis. High levels of miR-137 promoter methylation were associated with a significantly poorer disease-free survival rate (P=0.034), but were not associated with overall survival, in Kaplan-Meier analysis and univariate analysis. In conclusion, the results of the present study indicated that miR-137 is downregulated and that its promoter is aberrantly methylated in lung cancer, and that high levels of miR-137 promoter methylation may have prognostic value for poor disease-free survival.

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*Key words:* clinicopathological characteristics, microRNA-137, non-small cell lung cancer, prognostic value, promoter methylation

## Introduction

Lung cancer is the most frequently diagnosed malignancy and the leading cause of cancer-associated mortality worldwide (1). Non-small cell lung cancer (NSCLC) accounts for 85-90% of all lung cancer cases (2). Patients diagnosed with symptomatic lung cancer have a poor prognosis, with an overall 5-year survival rate of 16% in the United States (3).

Lung carcinogenesis is a multistep process associated with the activation of oncogenes and the inactivation of tumor-suppressing genes (4). However, the molecular mechanisms underlying the progression of NSCLC remain poorly understood. Therefore, there is an urgent requirement for further investigation of the mechanisms that lead to the development and progression of lung cancer in order to identify novel biomarkers and therapeutic targets.

MicroRNAs (miRNAs) are small, non-coding RNAs ~22 nucleotides in length that regulate gene expression by either degradation or repression of mRNA translation (5). MicroRNAs serve essential roles in a variety of biological processes, including cell death, differentiation, proliferation and metabolism (5,6). Altered miRNA expression occurs in numerous types of human cancer (7) and is associated with the initiation and progression of cancer (8). The expression of miRNAs may be controlled through epigenetic mechanisms, with ~10% of miRNAs being regulated by DNA methylation (9).

MicroRNA-137 (miR-137) is located on human chromosome 1p21.3 and is embedded in a CpG island (10,11). miR-137 is frequently downregulated in several types of cancer,

including colorectal cancer, gastric cancer, glioblastoma and NSCLC (12-16). Previous studies have suggested that miR-137 silencing may be the result of hypermethylation of the miR-137 gene promoter (11,15,16). miR-137 promoter methylation is associated with poor prognosis in certain types of cancer, including gastric cancer (17) and squamous cell carcinoma of the head and neck (18). A previous study by Kang *et al* demonstrated that the level of miR-137 promoter methylation was significantly higher in lung tumors than in the adjacent non-tumor tissues (19). However, the aforementioned study did not assess whether miR-137 promoter methylation has prognostic value for recurrence or overall survival in lung cancer (19).

The principal aim of the present study was to evaluate whether methylation of the miR-137 promoter represents a prognostic biomarker for overall and disease-free survival in NSCLC. The overall objective of the present study was to provide an experimental and theoretical basis for further study of the associations between miR-137 promoter methylation and prognosis in NSCLC.

## Materials and methods

*Ethical approval*. The present study was approved by the Ethics Committee of Subei People's Hospital (Yangzhou, China) and written informed consent was obtained from all participants.

*Study population*. A total of 10 pairs of 4% formalin-fixed for 24 h at room temperature, paraffin-embedded (FFPE) NSCLC tissues and corresponding matched non-tumor lung tissues were collected (from 4 patients with squamous cell carcinoma and 6 patients with adenocarcinoma) between May 2012 and October 2012, at Subei People's Hospital. A further 56 FFPE NSCLC tissues (from 31 patients with squamous cell carcinoma and 25 patients with adenocarcinoma) were obtained between February 2008 and December 2009. All tissue samples were collected prior to treatment with chemoradiotherapy. Each sample was confirmed by histopathological evaluation using hematoxylin and eosin staining. Clinical data were recorded at the time of resection and patients were prospectively followed-up to ascertain vital status (the last follow-up date was April 30, 2012).

*Cell culture*. Human lung cancer A549 and H1299 cells and normal bronchial epithelial BEAS-2B cells were purchased from the Cell Resource Center, Shanghai Institute of Biochemistry, China. (http://www.sibcb.ac.cn/) BEAS-2B cells were derived by transforming human bronchial epithelial cells with an adenovirus 12-simian virus 40 construct, as previously described (20). The cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (both from Wisent, Inc., St. Bruno, QC, Canada) at 37˚C in a humidified air atmosphere containing 5% carbon dioxide.

*Treatment with 5-aza-2'-deoxycytidine*. A549 and H1299 cells were seeded onto 24-well plates on day 0, exposed to the DNA methylation inhibitor 5-aza-2'-deoxycytidine (5-aza-dC; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at a final concentration of 5 $\mu$mol/l between day 1 and day 3 at 37˚C in a humidified air atmosphere containing 5% $CO_2$ and were harvested under Trypsin-EDTA digestion harvested for RT-qPCR analysis of miR-137 expression on day 4.

*RNA isolation*. Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Total tissue RNA was extracted from FFPE tissue sections using the miRNeasy FFPE kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's protocol. Paraffin was removed from freshly cut FFPE tissue sections each up to 10-$\mu$m thick using deparaffinization solution using the miRNeasy FFPE kit (Qiagen, Inc.), and samples under went protease digestion at room temperature to release RNA from the sections, then short incubation (at 56˚C for 15 min, then at 80˚C for 15 min) to reverse formalin cross-linking of the released nucleic acids and DNase digestion to remove DNA. Total RNA (including miRNAs) was dissolved in 20 $\mu$l RNase-free water. RNA concentrations were measured using a NanoDrop-1000 (Thermo Fisher Scientific, Inc.) and RNA integrity was determined by 1.5% agarose gel electrophoresis.

*Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis of miRNA-137 expression*. RNA was reverse transcribed using the RevertAid First Strand cDNA kit (Thermo Fisher Scientific, Inc.) according to the manufacture's protocolin combination with a stem-loop primer for miRNA-137U6 small nuclear RNA was used as an internal control to normalize the expression levels of miRNA-137. The primer sequences are presented in Table I. Briefly, 1.0 $\mu$g total RNA was combined with 4.0 $\mu$l Ribo Lock RNase inhibitor, 2.0 $\mu$l dNTP mix (10 mM each) and 1.0 $\mu$l RevertAid M-MuLV reverse transcriptase in a total reaction volume of 20 $\mu$l, which was incubated on an ABI Prism 7900HT Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) at 25˚C for 5 min, 42˚C for 60 min and 70˚C for 5 min.

RT-qPCR was performed using LightCycler® 480 SYBR-Green I Master mix on a LightCycler® 480 Real-Time PCR system (both Roche Diagnostics, Basel, Switzerland). Each 10 $\mu$l PCR mixture contained 1 $\mu$l 20-fold diluted reverse transcription product, 5 $\mu$l SYBR-Green Master mix, 2 $\mu$l RNase-free water, and 1 $\mu$l forward and reverse primers. The reactions were incubated at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 65˚C for 60 sec. Relative miRNA-137 expression was calculated using the $2^{-\Delta\Delta Cq}$ method (21), where $\Delta Cq$ is the difference in threshold cycles (Cq) for the target and reference = Cq(miRNA-137)-Cq(U6). RT and PCR primers were synthesized by Shanghai Sheng Gong Biology Engineering Technology Service, Ltd. (Shanghai, China).

*Analysis of miR-137 promoter lesion and sodium bisulfate conversion*. The miR-137 CpG is lands were identified using EMBOSS Software Version 6.3.1 (Institut Pasteur, Paris, France) and EMBOSS (http://gensoft.pasteur.fr/docs/EMBOSS/6.3.1/). Total tissue DNA was extracted from FFPE tissue scrolls using the Qiagen EpiTect Plus FFPE Bisulfite kit (Qiagen, Inc.) according to the manufacturer's protocol. FFPE tissue scrolls were deparaffinized, followed by proteinase digestion and de-cross-linking as aforementioned in RNA isolation

Table I. Primer sequences.

| Primer | Sequence (5'-3') |
|---|---|
| miRNA reverse transcription primer sequence | |
| miRNA-137 | TTATTGCTTAAGAATACGCGTAG |
| U6 snRNA | AAAATATGGAACGCTTCACGAATTTG |
| qPCR primer sequence | |
| miRNA-137 forward | CAAGGCTTGTTAACACTGTAAC |
| miRNA-137 reverse | TCTGTCAATGTCTGAATAAATG |
| U6 snRNA forward | CTCGCTTCGGCAGCACATATACT |
| U6 snRNA reverse | ACGCTTCACGAATTTGCGTGTC |
| MS-qPCR primer sequence | |
| Methylated alleles forward | 5'-GCGGTAGTAGTAGCGGTAGC-3' |
| Methylated alleles reverse | 5'-ACCCGTCACCGAAAAAAA-3' |
| Unmethylated alleles forward | 5'-GGTGGTAGTAGTAGTGGTAGT-3' |
| Unmethylated alleles reverse | 5'-TACCCATCACCAAAAAAAA-3' |

qPCR, quantitative polymerase chain reaction; MS, methylation specific; miRNA, microRNA; snRNA, small nuclear RNA.

paragraph. The DNA bisulfite reaction was then set up and performed using an ABI Prism 7900HT Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using the Qiagen EpiTect Plus FFPE Bisulfite kit (Qiagen, Inc.) according to the manufacturer's protocol. Upon completion of the bisulfite conversion, modified DNA was purified and eluted, the DNA concentrations were measured using a NanoDrop-1000 (Thermo Fisher Scientific, Inc.) and the samples were stored at -20˚C for further analysis.

*Methylation-specific (MS) qPCR*. The methylation status of the miR-137 promoter in the FFPE tissue sample was determined by MS-qPCR, as previously described (19). Modified DNA (10 ng) was subjected to PCR amplification on an ABI Prism 7900HT Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) at 90˚C for 5 min, followed by 40 cycles of 95˚C for 15 sec, 60˚C for 30 sec and 72˚C for 15 sec, then a 10 min final extension at 72˚C. The PCR products were diluted 500-fold with water, and 1 $\mu$l aliquots were subjected to MS-qPCR using a LightCycler® 480 SYBR-Green I Master (Roche Diagnostics) at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 51˚C for 60 sec, calculated with the formula $2^{-\Delta Cq}$[Cq(methylated)-Cq (unmethylated)] (21), and expressed as a percentages. qPCR primer sequences are listed in Table I.

*Statistical analysis*. Data were analyzed using the SPSS 18.0 statistical software (SPSS, Inc., Chicago, IL, USA). Values presented as the mean ± standard deviation and were analyzed using one-way analysis of variance with post hoc analysis by least significant difference (LSD) test. The correlation between miR-137 expression and tissue methylation levels was evaluated using Pearson's correlation analysis. Descriptive statistics were used to compare the demographic and clinicopathological characteristics (sex, age, pathology, smoking, status, tumor size, histologic grade, T category, lymph node metastasis and clinical stage) (22) of the study population stratified by the miR-137 promoter methylation status, and categorical variables were compared using analysis of variance with post hoc analysis by LSD test. The univariate Kaplan-Meier method was used to estimate disease-free survival and overall survival rates, and survival differences were compared using the log-rank test. In analysis of disease-free and overall survival rates, patients who succumbed prior to recurrence were considered censored at the point of mortality. A multivariable Cox proportional hazards model was used to assess the prognostic value of the level of miR-137 promoter methylation for disease-free and overall survival rates, following adjustment for sex, age, histological grade, T category, age and smoking status. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*miR-137 is down regulated in lung cancer cell lines and human tumor tissues*. The expression of miR-137 was determined by qRT-PCR (Fig. 1). miR-137 was markedly downregulated in lung cancer A549 and H1299 cells compared with that in normal lung bronchial epithelial BEAS-2B cells (Fig. 1A). In line with the cell line analyses, the expression of miR-137 was significantly downregulated in the 10 FFPE lung cancer tissue specimens compared with that in the paired adjacent normal lung tissues ($P=0.037$; Fig. 1B and C).

*Promoter hypermethylation downregulated miR-137 in lung cancer cell lines and human tumor tissues*. CpG plot EMBOSS Software version 6.3.1 software (http://gensoft.pasteur.fr/docs/EMBOSS/6.3.1/; Institut Pasteur, Paris, France) identified 2 CpG islands located close to the miR-137 gene (Fig. 1D) and promoter hypermethylation has previously been reported to be responsible for the repression of miR-137 (14). Therefore, miR-137 expression was analyzed in a lung cell line treated with the methyltransferase inhibitor 5-aza-CdR (5 $\mu$mol/l). Untreated control cells expressed lower

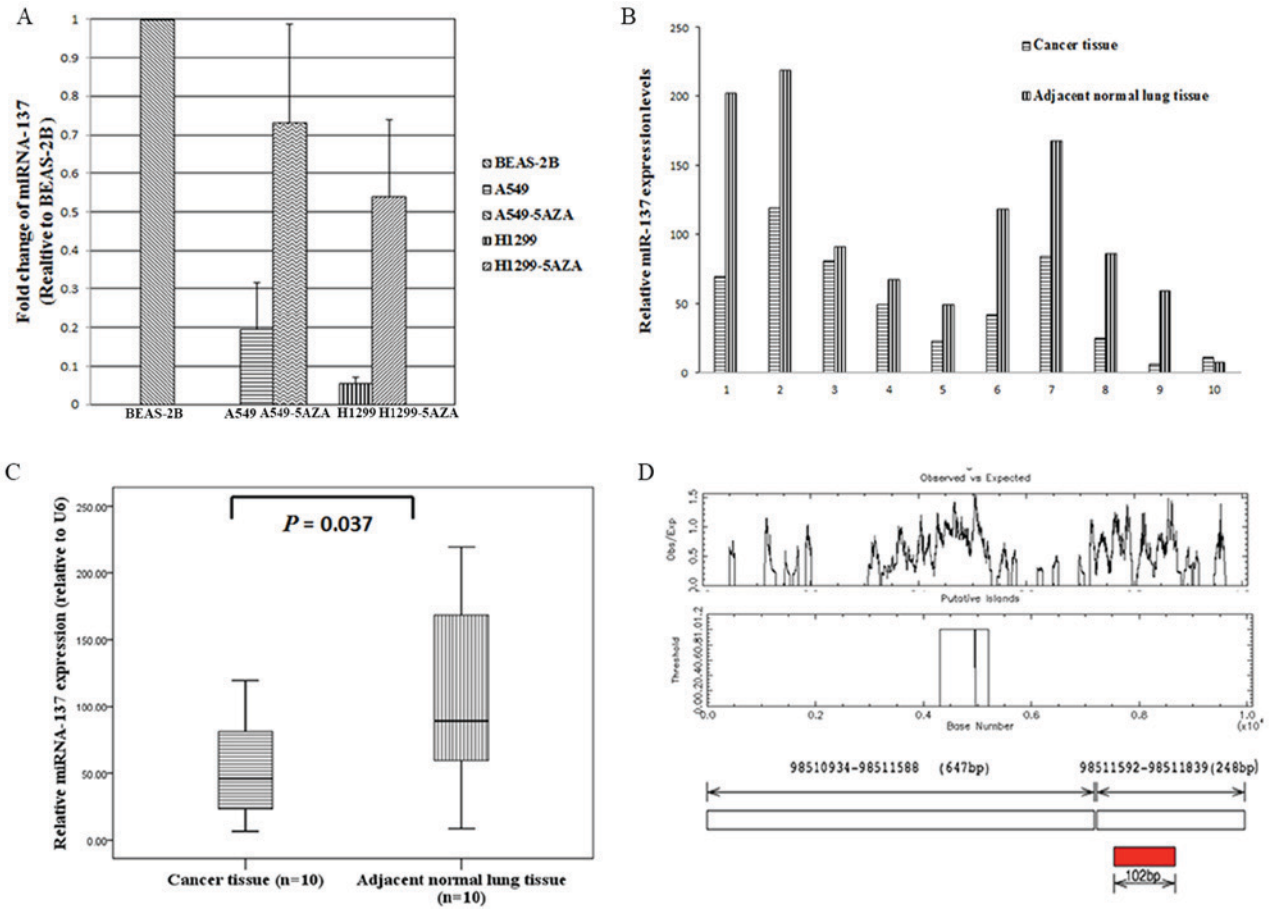


Figure 1. miRNA-137 is downregulated in lung cancer cell lines and clinical specimens. (A) The relative expression of miRNA-137 in A549 and H1299 lung adenocarcinoma cells and BEAS-2B normal bronchial epithelial cells was determined by RT-qPCR assay and normalized to U6. Values a represented as fold-changes relative to BEAS-2B cells. Results are presented as the mean ± standard deviation of 3 independent experiments. (B) Relative expression of miR-137 in 10 paired lung cancer tissues and the corresponding non-tumor lung tissues was determined by RT-qPCR assay and normalized to U6 with (C) quantification. miRNA, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction. (D) Illustration of CpG site in miR-137. miRNA, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

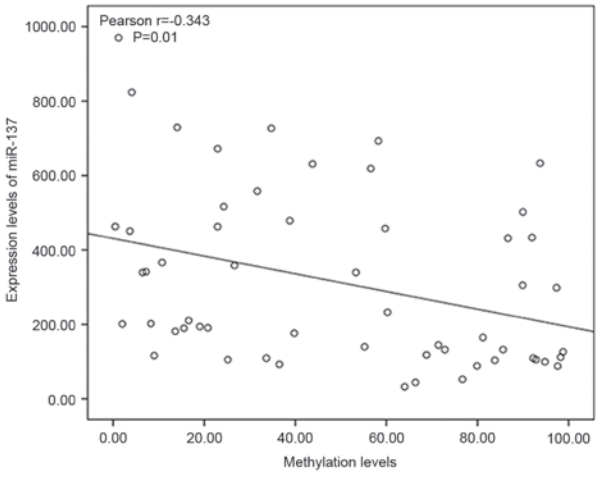


Figure 2. miR-137 promoter methylation is significantly negatively correlated with miR-137 expression in lung cancer tissues (n=56; r=-0.343, P=0.01; Pearson's correlation). miR, microRNA.

levels of miR-137, whereas 5-aza-CdR-treated cells expressed higher levels of miR-137 (Fig. 1A). The methylation status of the CpG sites was consistent with the levels of miR-137 expressed in the lung cancer cell lines.

Subsequently, miR-137 expression and the promoter methylation status in the 56 FFPE lung cancer tissues were compared using RT-qPCR and MS-qPCR. A significant negative correlation between the expression level of miR-137 and miR-137 promoter methylation was observed in the lung cancer tissues (Pearson's correlation, r=-0.343, P=0.01; Fig. 2), suggesting that promoter methylation silences miR-137 in lung cancer.

*miR-137 promoter methylation status is associated with lymph node metastasis and advanced clinical stage*. To determine whether the miR-137 promoter methylation status is associated with lung cancer, the association between the miR-137 promoter methylation status and the clinicopathological characteristics of lung cancer were further analyzed. Low levels of miR-137 promoter methylation were significantly associated with smoking, positive lymph node metastasis and advanced clinical stage (P=0.027, P=0.004 and P=0.021, respectively) (Table II). There was no significant association between miR-137 promoter methylation and sex, age, pathology, tumor size, histological grade or T category (Table II).

*High levels of miR-137 promoter methylation are associated with poor disease-free survival*. Kaplan-Meier survival

Table II. Characteristics of 56 patients with non-small cell lung cancer by microRNA-137 promoter methylation levels.

| Variable | No. | Mean ± SD | P-value[a] |
|---|---|---|---|
| Sex | | | 0.773 |
| Male | 43 | 49.2±30.1 | |
| Female | 13 | 51.8±25.6 | |
| Age (years) | | | 0.508 |
| <60 | 24 | 46.8±29.0 | |
| ≥60 | 32 | 52.0±29.0 | |
| Smoking status | | | 0.027 |
| 0 | 18 | 35.8±21.4 | |
| <20 pack/year | 13 | 50.5±37.6 | |
| ≥20 pack-y | 25 | 59.5±25.2 | |
| Histiotype | | | 0.643 |
| Adenocarcinoma | 25 | 48.1±27.6 | |
| Squamous cell | 31 | 51.8±30.8 | |
| T-status | | | 0.610 |
| T1 | 30 | 46.9±28.5 | |
| T2 | 21 | 51.4±31.9 | |
| T3 | 5 | 60.2±17.0 | |
| N-status | | | 0.004 |
| N0 | 31 | 38.9±26.5 | |
| N1 | 14 | 60.6±29.9 | |
| N2 | 11 | 66.8±21.5 | |
| TNM | | | 0.021 |
| I | 26 | 38.8±28.1 | |
| II | 17 | 57.8±27.1 | |
| III | 13 | 61.7±26.2 | |
| Differentiation | | | 0.621 |
| Well | 11 | 50.6±27.1 | |
| Moderately | 34 | 51.9±29.6 | |
| Poorly | 11 | 42.1±29.7 | |

[a]As determined by one-way analysis of variance. SD, standard deviation; T, tumor; N, node; TNM, Tumor-Node-Metastasis; SD, standard deviation; T, tumor; N, node; TNM, Tumor-Node-Metastasis.

analysis and multivariate Cox proportional hazards analysis were employed to evaluate the association between miR-137 promoter methylation and prognosis in NSCLC. In the present study, 1 patient succumbed to pneumonia shortly after surgery and 8 patients were lost to follow-up. Patients were stratified according to the median relative miR-137 promoter methylation level (43.79%) in the tumor specimens from the remaining 47 tumor tissues; low miR-137 promoter methylation (n=24; ≤median) and high miR-137 promoter methylation group (n=23; >median). In univariate Kaplan-Meier analysis, patients with high levels of miR-137 promoter methylation exhibited significantly poorer disease-free survival rates (P=0.034; Fig. 3A), but no significant difference was observed in overall survival rates (P=0.136; Fig. 3B), compared with patients with low levels of miR-137 promoter methylation.

Table III. Cox proportional hazards model analysis of adjusted hazard ratios for progression-free and overall survival rates according to miRNA-137 promoter methylation levels in NSCLC patients.

| miR-137 promoter methylation levels | HR | 95% CI | P-value |
|---|---|---|---|
| Progression-free survival | | | |
| Low levels | 1.000 | | |
| High levels | 3.333 | 1.108-10.029 | 0.032 |
| Sex | 0.298 | 0.061-1.453 | 0.134 |
| Age | 1.081 | 0.302-3.866 | 0.904 |
| Smoking status | 1.359 | 0.678-2.726 | 0.387 |
| Histiotype | 2.255 | 0.554-9.172 | 0.256 |
| T-stage | 0.044 | 1.021-4.919 | 0.044 |
| Overall survival | | | |
| Low levels | 1.000 | | |
| High | 2.537 | 0.934-6.890 | 0.366 |
| Sex | 0.341 | 0.070-1.655 | 0.182 |
| Age | 1.503 | 0.407-5.548 | 0.541 |
| Smoking status | 1.439 | 0.711-2.913 | 0.311 |
| Histiotype | 2.497 | 0.572-10.983 | 0.572 |
| T-stage | 1.554 | 0.781-3.091 | 0.209 |

NSCLC, non-small cell lung cancer; HR, hazard ratio; CI, confidence interval; T, tumor.

In the multivariable Cox proportional hazards analysis, adjusting for gender, age, histologic grade, smoking status and T stage, the results showed that patients with high levels of miR-137 promoter methylation had a higher risk of disease-free death (hazard ratio, 3.333; 95% CI, 1.108-10.028, P=0.032, Table III) compared with the patients with low levels of miR-137 promoter methylation; levels of miR-137 promoter methylation was not significantly associated with overall survival (P=0.366).

## Discussion

The aim of the present study was to investigate whether miR-137 promoter hypermethylation is associated with overall survival and disease-free survival in lung cancer. Although miR-137 has been reported to exert tumor-suppressor activity in various types of cancer (12-18), there is limited data on the function of miR-137 in NSCLC. The present study demonstrated that miR-137 is downregulated in NSCLC cell lines and tumor tissues.

Several factors may reduce the expression of miRNAs. DNA methylation of CpG islands is an important regulatory mechanism for gene expression, which has also been revealed to be responsible for inactivating the expression of miRNAs, including that of miRNA-137 (19). miR-137 is downregulated in the tissues of several types of cancer compared with normal tissues (11-16,23) and functions as a tumor suppressor by targeting several genes, including cyclooxygenase-2, cell division protein kinase 6, cell division control protein

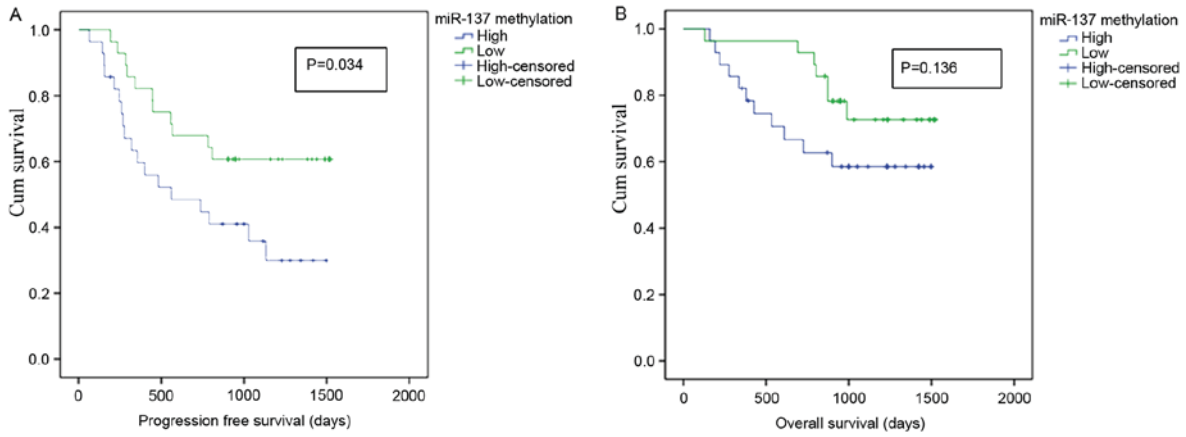


Figure 3. Survival analysis. Kaplan-Meier curves for (A) disease-free survival and (B) overall survival curves for patients with non-small cell lung cancer stratified by the level of miRNA-137 promoter methylation. Survival data were compared using the log-rank test. Patients with low levels of miRNA-137 promoter methylation exhibited shorter disease-free and overall survival times than patients with high levels of miRNA-137 promoter methylation. miRNA, microRNA.

42 homolog, C-terminal-binding protein 1, estrogen-related receptor, KIT proto-oncogene receptor tyrosine kinase, glioma pathogenesis-related protein-1, paxillin and solute carrier family 22 member 8 (12-15,24-29). Treatment with the DNA methytransferase inhibitor 5-aza-dC increased miR-137 expression in A549 and H1299 cells, suggesting that promoter methylation may be one mechanism that leads to the silencing of miR-137 in lung cancer, which is consistent with the study of Kang *et al* (19). In agreement with this hypothesis, the level of miR-137 promoter methylation was significantly correlated with miR-137 expression in the 56 human lung cancer tissues tested in the present study.

In line with the results of a study undertaken by Zhang *et al* (12) on non-small-cell lung cancer, low levels of miR-137 promoter methylation were significantly associated with smoking, positive lymph node status and advanced Tumor-Node-Metastasis stage in human NSCLC, but not with tumor size, tumor status, sex, differentiation or histological type. Furthermore, to the best of our knowledge, the present study provides the first evidence that high levels of miR-137 promoter methylation are associated with poor disease-free survival, and multivariate Cox proportional hazards analysis demonstrated that miR-137 promoter methylation was an independent prognostic factor for disease-free survival.

The present study has a number of limitations. To begin with, the role of miR-137 as a tumor-suppressor in lung cancer was not confirmed. Secondly, further research is required to investigate the environmental and personal risk factors associated with miR-137 promoter methylation. Finally, due to the relatively small sample size and short follow-up in the present study, further research is required to confirm the association between miR-137 promoter methylation and survival outcomes in NSCLC.

In conclusion, aberrant miR-137 promoter methylation is a common feature in NSCLC. Further studies should focus on the quantitative assessment of miR-137 promoter methylation in tumor tissues and specific types of lung cancer, with the aim of developing etiological and prognostic markers to prolong survival in lung cancer.

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**Availability of data and materials**

The datasets used or analyzed during the present study are available from the corresponding author on reasonable request.

**Author's contributions**

Conceptualization, LM, FW and XX. Formal analysis, LM. Funding acquisition, LM and YC. Methodology, SL, YC and JY. Resources, LM and SH. Software, LM and SL. Supervision, XX and SH. Analysis and interpretation of data, SH. Writing-original draft, LM. Writing-review and editing, XX.

**Ethics approval and consent to participate**

The present study was approved by the Ethics Committee of Subei People's Hospital (Yangzhou, China) and written informed consent was obtained from all participants.

**Consent for publication**

Written informed consent was obtained from all participants.

**Competing interests**

The authors declare that they have no competing interests.

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