DNA Methylation-Mediated Repression of miR-886-3p Predicts Poor Outcome of Human Small Cell Lung Cancer

Jianzhong Cao2, Yongmei Song1, Nan Bi2, Jie Shen2, Wenyang Liu2, Jing Fan1, Guogui Sun2, Tong Tong1, Jie He3, Yuankai Shi4, Xun Zhang5, Ning Lu2, Yinghua He5, Hongyu Zhang7, Kelong Ma7, Xiaoying Luo7, Lei Lv8, Hui Deng5, Jing Cheng5, Jingde Zhu0105, Luhua Wang, Qimin Zhan1

Abstract
Small cell lung cancer (SCLC) is one of the most aggressive types of cancer, yet the pathologic mechanisms underlying its devastating clinical outcome remain elusive. In this report, we surveyed 924 miRNA (miR) for their expressions in the formalin-fixed paraffin-embedded specimens from 42 patients with SCLC, and found that the downregulated miR-886-3p is closely correlated with the shorter survival of SCLC. This correlation was validated with another 40 cases. It was further discovered that loss of miR-886-3p expression was mediated by DNA hypermethylation of its promoter in both cultured SCLC cells and tumor samples. Moreover, miR-886-3p potently repressed cell proliferation, migration, and invasion of NCI-H446 cell in cell culture via suppression of the expression of its target genes: PLK1 and TGF-β1 at posttranscription levels. Forced upregulation of miR-886-3p greatly inhibited in vivo tumor growth, bone/muscle invasion, and lung metastasis of NCI-H446 cells. This newly identified miR-886-3p-PLK1/TGF-β1 nexus that modulates SCLC aggression suggests that both loss of miR-886-3p expression and hypermethylation of the miR-886-3p promoter are the promising indicators for poor outcome of as well as new therapeutic targets for SCLC. Cancer Res; 73(11); 3326–35. ©2013 AACR.

Introduction
Lung cancer is one of the most devastating types of cancer and the leading cause of cancer mortality. Small cell lung cancer (SCLC) accounts for 15% to 25% of all diagnosed lung cancers and attributes to approximate 200,000 deaths each year worldwide (1). Clinically, it is distinguished from non–small cell lung cancer (NSCLC) by rapid tumor growth and early onset of metastases. SCLC is thought to carry a variety of molecular abnormalities, including the activation of the Myc and K-RAS oncogenes and inactivation of the p53, Rb, and FHIT tumor suppressor genes (2). However, the underlying mechanisms by which SCLC manifests rapid progression remain to be defined.

Authors' Affiliations: 1State Key Laboratory of Molecular Oncology; Departments of 2Radiation Oncology, 3Thoracic Surgery, 4Medical Oncology, and 5Pathology, Cancer Hospital and Cancer Institute, Chinese Academy of Medical Sciences & Peking Union Medical College; 6National Engineering Research Center for Biochip Technology, Beijing; 7Shanghai Cancer Institute, Shanghai; and 8Cancer Epigenetic Laboratory, Anhui Cancer Hospital, Hefei, China

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

J. Cao, Y. Song, and N. Bi contributed equally to this work.

Corresponding Authors: Qimin Zhan, The State Key Laboratory of Molecular Oncology, Cancer Hospital and Cancer Institute, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100021, China. Phone: 86-10-67782694; Fax: 86-10-67715058; E-mail: zhangqm@pmc.edu.cn; Luhua Wang, The Department of Radiation Oncology, Cancer Hospital and Cancer Institute, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100021, China. E-mail: wfwsq@yahoo.com; and Jingde Zhu, Cancer Epigenetics Lab, Anhui Cancer Hospital, or Shanghai Cancer Institute. E-mail: zhu0105@vip.163.com
doi: 10.1158/0008-5472.CAN-12-3055
©2013 American Association for Cancer Research.
association of the loss of miR-886-3p expression with the poor clinical outcome of patients with SCLC, such an association was further confirmed by quantitative reverse transcriptase PCR (qRTPCR) analysis of another 40 patients’ specimens. We further showed that the downregulation of miR-886-3p expression is a result of hypermethylation of its promoter, suggesting the predictive value of both loss of expression of miR-886-3p and hypermethylation of miR-886 promoter for the poor survival of SCLC. We have also shown that miR-886-3p tumor suppression function is largely via its repression of the expression of the PLK1/TGF-β1 nexus at posttranscription level.

Materials and Methods

Clinical tissue samples and immunohistochemical analysis

The FFPE specimens of 82 cases with histologically confirmed limited-stage SCLC, Eastern Cooperative Oncology Group (ECOG) performance status of 0 to 1, and available clinical information as well as follow-up information in the Cancer Hospital, Chinese Academy of Medical Sciences (Beijing, China: Table 1). All the patients underwent surgical resection followed by adjuvant chemotherapy according to the standard of care. This study was approved by the Institutional Review Board of the Cancer Institute and Hospital, Chinese Academy of Medical Sciences.

The immunohistochemical analyses to evaluate PLK1 expression in FFPE specimens were carried out as previously described (28). Visible brown granules in the cytoplasm were determined as positive staining. Clinical SCLC specimens were reviewed with staining intensity and extent. Staining intensity was rated as: negative (0), bordering (1), weak (2), and strong (3). Staining extent was rated on the basis of the percentage of positive cells among all cells in the field. Samples with no staining cells were rated as 0, those with less than 25% of cells stained were rated as 1, those with 25% to 50% of cells stained were rated as 2, and those more than 50% of cells stained were rated as 3. The results of staining intensity and extent gave rise to an overall staining score. The samples that scored 0 were marked as grade 0, 1 to 2 marked as grade 1, 3 to 4 marked as grade 2, and 5 to 6 marked as grade 3.

RNA preparation and analysis

Low molecular weight RNA was isolated from TRIzol-extracted total RNA from both cultured cells and FFPE specimens using a polyethylene glycol (PEG) solution precipitation method (29), and was labeled using the T4 RNA ligase labeling method described by Thomson and colleagues (30). Hybridization was carried to a miRNA microarray panel (CapitalBio), which includes probes in triplicate for 924 mature human and mouse miRNA sequences and 8 short oligonucleotides that possessed no homology to any known RNA sequence as a control. The hybridized arrays were scanned with a LuxScan 10K-A laser confocal scanner, and the images obtained were then analyzed using the LuxScan 3.0 software (CapitalBio; ref. 4). The average values of the replicate spots of each miRNA were background subtracted. Signals were normalized using the median center tool for genes in the table.

Table 1. Clinical characteristics of the patients with limited-stage disease SCLC according to miR-886-3p levels

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patients with high-miR886-3p levels</th>
<th>Patients with low-miR886-3p levels</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>58 (33–77)</td>
<td>61 (39–76)</td>
<td>0.678</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (%)</td>
<td>15 (71.4)</td>
<td>15 (71.4)</td>
<td>1.000</td>
</tr>
<tr>
<td>Female (%)</td>
<td>6 (28.6)</td>
<td>6 (28.6)</td>
<td></td>
</tr>
<tr>
<td>ECOG performance status*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>7 (33.3)</td>
<td>1 (4.8)</td>
<td>0.045</td>
</tr>
<tr>
<td>1</td>
<td>14 (66.7)</td>
<td>20 (95.2)</td>
<td></td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>17 (81.0)</td>
<td>13 (61.9)</td>
<td>0.306</td>
</tr>
<tr>
<td>Nonsmokers (%)</td>
<td>4 (19.0)</td>
<td>8 (38.1)</td>
<td></td>
</tr>
<tr>
<td>Test group (N = 40)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>56 (36–73)</td>
<td>54 (33–73)</td>
<td>0.735</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (%)</td>
<td>14 (70.0)</td>
<td>18 (80.0)</td>
<td>0.235</td>
</tr>
<tr>
<td>Female (%)</td>
<td>6 (30.0)</td>
<td>2 (10.0)</td>
<td></td>
</tr>
<tr>
<td>ECOG performance status*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1 (5.0)</td>
<td>4 (20.0)</td>
<td>0.342</td>
</tr>
<tr>
<td>1</td>
<td>19 (95.0)</td>
<td>16 (80.0)</td>
<td></td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>13 (65.0)</td>
<td>16 (80.0)</td>
<td>0.480</td>
</tr>
<tr>
<td>Nonsmokers (%)</td>
<td>7 (35.0)</td>
<td>4 (20.0)</td>
<td></td>
</tr>
<tr>
<td>Total (N = 82)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, y</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>57 (33–77)</td>
<td>57 (33–76)</td>
<td>0.860</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (%)</td>
<td>29 (70.7)</td>
<td>33 (80.5)</td>
<td>0.304</td>
</tr>
<tr>
<td>Female (%)</td>
<td>12 (29.3)</td>
<td>8 (19.5)</td>
<td></td>
</tr>
<tr>
<td>ECOG performance status*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>9 (22.0)</td>
<td>4 (9.8)</td>
<td>0.226</td>
</tr>
<tr>
<td>1</td>
<td>32 (78.0)</td>
<td>37 (90.2)</td>
<td></td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>30 (73.2)</td>
<td>29 (70.7)</td>
<td>0.806</td>
</tr>
<tr>
<td>Nonsmokers (%)</td>
<td>11 (26.8)</td>
<td>12 (29.3)</td>
<td></td>
</tr>
</tbody>
</table>

*Details of ECOG performance status are listed in the Supplementary Table S4.

Cluster 3.0 software before the unsupervised clustering with complete linkage was conducted. Uncentered Pearson correlation to reveal the underlying structure of the miRNA expression (31). HRs from univariate Cox regression analysis, a standard method in biostatistics for dealing with survival data, were used to identify which miRs were correlated with overall survival (OS) of the patients (19). A gene was considered statistically significant if the parametric P value was < 0.05. All the raw data have been deposited in a MIAME...
compliant database (ArrayExpress, Gene Expression Omnibus: GSM678225-GSM678266).

Quantitative miRNA RT-PCR was conducted using the stem-loop qRT-PCR method. The cDNA was prepared from 100 ng total RNA with the specific primer for miR-886-3p or U6 as the internal control. The oligos used included: miRNA common reverse primer, GTGCAGGGTCCGAGGT; miR-886-3p, CGCGGGTGCTTACTGACCCTT; miR-886-3p forward primer, CACGC-GGGTCTTACTGAC. With U6 forward primer, CTCGCT-TCGGACGACA; and U6 reverse primer, AAGCGTCCAGGAGTTGACTGAC, qRT-PCR was conducted on an ABI Prizm 7300 Sequence Detection System using LightCycler DNA Master SYBR Green I Mix (Roche Molecular Biochemicals) following the manufacturer's instructions. The level of miR-886-3p was determined using the 2−ΔΔCT method in the SDS 1.3 software (Chen and colleagues; ref. 32) and normalized with the level of the internal control, U6, plotted for intercellular comparison.

Cell culture, transfection, and analysis

Four small cell cancer cell lines, NCI-H69 [American Type Culture Collection (ATCC) no. HTB-119], NCI-H209 (ATCC no. HTB-172), NCI-H446 (ATCC no. HTB-171), and NCI-H524 (ATCC no. CRL-5831), were cultured in RPMI-1640 medium (Gibco BRL) supplemented with 10% FBS (Gibco BRL). The miR-886-3p mimic (Mimic) and unrelated mimic control (miR-NC) were purchased from Genechem and RiboBio, respectively. The mimic transfection using Lipofectamine 2000 (Invitrogen) was conducted as previously described (33).

pGL3-Plk1-3′-UTR and pGL3-TGF-β-3′-UTR were constructed by inserting the 3′-UTR regions (Supplementary Table S1) of Plk1 and TGF-β1 into the XbaI sites of pGL3-control plasmid. PGL3-Plk1-3′-UTRmut harbors a substitution of 8 nucleotides within the core-binding region of the PLK1 3′-UTR, and the pGL3-TGF-β-3′-UTRmut carried a substitution of 7 nucleotides in the binding sites of TGF-β1. NCI-H446 cells were transfected with 50 ng of each reporter construct per 10 ng pRenilla (the control for transfection) and 100 nmol/L of Mimic or miR-NC, respectively. Both luciferase activities were measured in the cellular extracts 48 hours after transfection with Dual-Luciferase Reporter Assay System (Promega) and normalized with the Renilla luciferase activity, and then the relative luciferase activity between Mimic and miR-NC was calculated and plotted.

To determine the growth rate, 2 × 10^4 cells were seeded into 6-well plates 24 hours after transfection. Cells were harvested daily and counted in a hemocytometer for 4 days in triplicate.

Figure 1. Kaplan–Meier analyses of OS, PFS, and DMFS according to the miR-886-3p expression in SCLC. The microarray-based miR profiling was conducted in FFEP specimens of 42 SCLC cases (Training group) and validated by qRT-PCR in other 40 cases (Test group). The patients with SCLC were equally divided into high (top one-third), medium (middle one-third), and low (bottom one-third) groups. The OS, PFS, and DMFS were analyzed in the Training, Test, and Full group. A, summary table. B, OS in Training group. C, OS in Test group. D, OS for patients in both Training and Test groups (Full group). E, PFS (E) and DMFS (F) in full group.
DNA Methylation Regulates miR-886-3p in SCLC

Figure 2. DNA methylation status of the miR-886-3p promoter. A, the miR-886-3p gene. Vertical bars, the CpG dinucleotide in the miR-886 locus; arrows, the coding region of the miR-886-3p and CpG island. The regions analyzed by MSP and BSP are indicated. B, the sequence level detail of the miR-886 promoter region (7→202, 196 bp); the CpG dinucleotides within this region are numbered as 1 to 16. C, the methylation status of the miR-886-3p promoter in the normal lung tissues (Normal 1 and Normal 2) and NCI-H446 cell lines. The open and filled circles, the unmethylated and methylated CpGs, respectively. The location of the primers for BSP (BSPT) and MSP (MT, methylated allele; UT, unmethylated allele) are shown. D, the data in C was presented as percentages of the unmethylated/total CpGs in the plot. E, the methylated state by MSP. M, methylated state; U, unmethylated state; +, the positive controls for the methylated and unmethylated state, respectively; −, the no template control.

The Costar Transwell 8 μm inserts were coated with 50 μg reduced serum Matrigel (BD Biosciences) for the invasion assay according to the manufacturer’s instructions. RPMI-1640 was used to resuspend the cells at 48 hours posttransfection. Migration assays were conducted in the same manner excluding the Matrigel. After 12 hours, noninvasive cells and media were removed with a cotton swab. The underside of the transwell was stained with 0.5% crystal violet (Sigma). Ten replicates were counted. Inserts were selected with G418 (400 μg/mL) for 1 week and pooled for further studies.

DNA methylation analysis

Genome DNA was prepared from NCI-H446, H-209, H-524, and H-69 cell lines, SCLC clinical samples as well as lung tissues of 2 noncancerous patients. A bisulphite conversion was carried out with a modified form of Hayatsu protocol (35).

The CpG Island Searcher Program (36) was used to determine whether any CpG island existed within the miR-886 locus (1 kilobases upstream and downstream of the miR-886 coding region; ref. 37). Both bisulfite sequencing (BSP) and methylation-specific PCR (MSP) analyses for the methylation of the miR-886-3p gene was carried out as previously described (38, 39) with appropriate primers (Supplementary Table S2).

5-Aza-2′-deoxycytidine and TSA treatment of the NCI-H446 cell line

NCI-H446 cells were treated with 1.5 μmol/L 5-Aza-2′-deoxycytidine (Sigma A3656) for 2 or 4 days. Twenty-four hours before harvest, 0.5 μmol/L Trichostatin A (Sigma T8552) was added. DNA, RNA, and protein were extracted and analyzed for the methylation status of the miR-886 promoter, as well as expression of miR-886-3p and its targeted proteins. Cellular proteins were prepared and subjected to immunoblotting analysis as described previously (40).

Analysis of in vivo tumorigenicity

BALB/c nude mice, 4 weeks old, were provided by the Cancer Institute, Chinese Academy of Medical Sciences for the in vivo tumorigenicity study; the Institutional Animal Welfare Guidelines were followed. Mice were injected subcutaneously with 3 × 106 cells in 0.2 mL into the right top back and monitored for tumor volumes, overall health, and total body weight. The size of the tumor was determined by caliper measurement of the
Cox proportional hazard regression was then used to identify miRNAs that were correlated with the OS. Chi² test was used to compare PLK levels in different miR-886-3p expression groups. All P values were 2 sided, and a P < 0.05 was considered to be statistically significant. All the analyses were conducted using the SPSS software package (version 11.5, SPSS Inc.).

Results

Downregulation of miR-886-3p correlated with the poor clinical outcome of SCLC

To determine the miR’s roles in the clinic behavior of SCLC, we first used a microarray-based assay to profile 924 known miRs’ expression in FFPE specimens from 42 patients with SCLC (a Training set; Table 1), who were recruited in the Cancer Hospital, Chinese Academy of Medical Science, from 2002 to 2005. Among the 546 miRs with meaningful levels of signal (not shown), 2 downregulated miRs, miR-886-3p and miR-150, were found significantly associated with the shorter survival of SCLC. The miR-886-3p was chosen for further study. We quantified by qRT-PCR analysis the miR-886-3p in 10 randomly selected samples in the Training set and found a good concordance between the array data and qRT-PCR results (correlation coefficients: 0.85, P = 0.002). Patients were equally divided to 3 groups (14 cases/group): high (top one-third), moderate (middle one-third), and low (bottom one-third) expression groups and compared (Fig. 1A). The low miR-886-3p expression group had significantly shorter OS than the high expression group: the 5-year survival rate was 27.0% in the low expression group and 77.0% in the high expression group (P = 0.045; Fig. 1B).

We further validated this association in another 40 patients (the Testing set), for which the miR-886-3p level in FFPE specimens was qRT-PCR determined. Again, the low (13 cases) displayed shorter survival than in the high expression group (13 cases; Fig. 1A; the 5-year OS rate was 15.0% in the low vs. 77.0% in the high expression group, P < 0.0001; Fig. 1C).

The OS of the patients was significantly shorter in the low (27 cases) than in the high expression group (27 cases; P < 0.0001; Fig. 1D). Other 2 prognostics measurements, PFS and distance metastasis-free survival (DMFS), were also significantly worse in the low than in the high expression group (P = 0.001 in PFS and P = 0.0005 in DMFS, respectively; Fig. 1E and F and Supplementary Fig. S1A–S1E). Therefore, the down-regulation of miR-886-3p seemed to contribute to tumor progression and showed an outcome predictive value of SCLC.

The hypermethylation of the miR-886-3p promoter correlates with the low level of miR-886-3p expression in both SCLC cell lines and clinical samples

The miR-886-3p promoter is located in a typical CpG island, suggesting a possible involvement of DNA methylation in the regulation of miR-886-3p transcription (Fig. 2A). We carried a BSP analysis to determine the DNA methylation status of each of the 16 CpGs in the promoter region from −7 to −202, upstream of the miR-886-3p–coding region in NCI-H446 cells and normal lung tissues (Normal 1 and 2) from 2 patients with noncancerous lung disease (Fig. 2B). Although 93.8% and 100% of CpGs were unmethylated in Normal 1 and 2, only 8.1% of

Figure 3. Association of the promoter methylation status, level of expression of the miR-886-3p gene with the clinical outcome in SCLC. A, the MSP result of 6 representative samples. M, the MSP result by the primer pair for the methylated state of the promoter; U, result by the primer pair for the unmethylated state of miR-886-3p promoter. B, summaries for the DNA-methylated state of the promoter and expression of the miR-886-3p gene and the patient survival of patients with SCLC in this analysis (P = 0.001). C, Kaplan–Meier curve of estimated OS for patients with SCLC with different methylated states of miR-886-3p gene (P = 0.019).

Subcutaneous tumor mass. Tumor volumes were calculated according to the formula 4/3πr³ × r² (r¹ × r²). Each experimental group contained 7 mice, and the experiments were repeated 3 times (34).

Statistical analysis

Student t test or χ² test was used to calculate the difference in patient clinical characteristics. OS was calculated as the time to death from the date of diagnosis. Progression-free survival (PFS) was calculated as the time to progression or death without progression from the date of diagnosis. Local recurrence was defined as both ipsilateral pulmonary and mediastinal recurrences, whereas distant metastasis was defined as any recurrence occurring in the liver, contralateral lung, adrenal glands, brain, bone, or other locations. High miRNA expression was classified as the top one-third and low expression was classified as the bottom one-third in each sample group. Survival curves were estimated with the Kaplan–Meier method and compared with the log-rank test. Cox proportional
CpGs were unmethylated in NCI-H446 cells (Fig. 2C and D). The differential methylation of the miR-886-3p promoter was further confirmed by the MSP analysis (Fig. 2E). As shown in the figure, only the PCR product from the primer pair specific for the methylated state was evident in NCI-H446 cells (the methylated state), whereas the opposite was true in both normal lung tissues (the unmethylated state).

To further confirm the regulatory role of DNA methylation in miR-886-3p expression, we treated NCI-H446 cells for 2 or 4 days with a DNA methyltransferase inhibitor, 5-Aza-2'-deoxycytidine, and the DNA methylation status of the miR-886-3p promoter was determined by BSP (Supplementary Fig. S2A). Unmethylated CpG within this region were up from 8.1% in the mock-treated cells (Mock) to 20.8% (5-Aza/2 days) and 51.6% (5-Aza/4 days), respectively (Supplementary Fig. S2B and S2C). As expected, the miR-886-3p level was up by more than about 2.6-fold in the cells treated by 5-Aza for 4 days, whereas the level of other 3 CpG island-free miR genes, miR-17, miR-31, and miR-150, remained unchanged (Supplementary Fig. S2D). Furthermore, both PLK1 and TGF-β1 proteins, the 2 primary targets of miR-886-3p, were downregulated to about 50% of the level of the Mock treated (Supplementary Fig. S2E).

A compatible analysis was extended to 3 additional SCLC cell lines: NCI-H209, NCI-H524, and NCI-H69 cells. The miR-886-3p promoter was almost fully methylated in the former 2 (11.4 and 13% CpGs unmethylated) but essentially unmethylated in the NCI-H69 cell line (78.1% CpGs unmethylated; Supplementary Fig. S3A and S3B). As expected, the miR-886-3p level in NCI-H69 was 1.43- or 7.33-fold higher than in NCI-H209 and NCI-H524 cells, respectively (Supplementary Fig. S3C). Taken all together, the miR-886-3p expression was indeed under the negative control of the methylation status of its promoter.

Concerning the clinical relevance of the DNA methylation state of the miR-886-3p promoter, we further determined the methylation status of the miR-886-3p promoter by MSP in samples of 33 patient with known miR-886-3p expression levels: 17 cases in the low and 16 cases in the high expression groups (Fig. 3A–C and Supplementary Table S3).
miR-886-3p negatively regulated proliferation, migration, and invasion of NCI-H446 cells by repressing the expression of its targets: PLK1 and TGF-β1

We further showed that a forced elevation of the miR-886-3p level in NCI-H446 cells by miR-886-3p mimic (Mimic) transfection (Fig. 4A) repressed proliferation (Fig. 4B), migration, and invasion (Fig. 4C and D), suggesting a tumor suppressor function of miR-886-3p. Taking both confidence score and their known roles in carcinogenesis into consideration, mRNA of the following 3 candidates, PLK1, TGF-β1, and MYBBP1A, predicted by TargetScan4.0 software were assessed by qRT-PCR in the Mimic-transfected versus miR-NC–transfected cells. Significantly, the Mimic transfection decreased the levels of both mRNAs (Fig. 4E) and proteins (Fig. 4F) of PLK1 and TGF-β1, but not significantly the mRNA of MYBBP1A (Fig. 4E). To further investigate their role as a true target for miR-886-3p, the wild-type or mutant 3'-UTR regions of PLK1 and TGF-β1 genes were placed at the downstream of the luciferase reporter gene, to create pGL3-Pik1-3'-UTR, pGL3-Pik1-3'-UTRmut, pGL3- TGF-β1-3'-UTR, and pGL3-TGF-β1-3'-UTRmut constructs, respectively (Fig. 5A and B). Each construct was cotransfected with Mimic or miR-NC, and luciferase activity was quantified and compared. As expected, the luciferase activities of both pGL3-Pik1-3'-UTR and pGL3-TGF-β1-3'-UTR (Fig. 5C), but not their mutant counterparts (Fig. 5D), were decreased about a half by Mimic in comparison with the Mock. It is concluded that the tumor suppressor function of miR-886-3p was likely achieved via its repression on both PLK1 and TGF-β1 expression at both translation and stability levels of mRNA.

A further piece of evidence came from the following observation where the PLK1 and TGF-β1 proteins (endogenous) in NCI-H446 cells were abolished by Mimic transfection and recovered after 24 hours by a transfection of both 3'-UTR–free pEGFP-N1-PLK1 and pEGFP-N1-TGFβ1 expression constructs, respectively. Consequently, all the phenotypic features; the compromised cell proliferation and migration/invasion created by Mimic transfection, were reversed by transfection of both expression constructs (Supplementary Fig. S4A–S4C).

Figure 5. miR-886-3p-repressing activities targeted the 3'-UTR of both PLK1 and TGF-β1. A, sequence pairing of miR-886-3p to the binding sites on the 3'-UTR regions of PLK1 and TGF-β1. B, the luciferase reporter constructs with the wild-type 3'-UTR regions or mutated 3'-UTR regions of PLK1 and TGF-β1 involved. The mutated sequences are in italic. C, Mimic’s effects on the luciferase activity of the transfected PLK1 reporter constructs. The relative fold luciferase activity of the transfected NCI-H446 cells was calculated and plotted. D, Mimic’s effects on the luciferase activity of the transfected TGF-β1 reporter constructs.
To confirm the clinical relevance of the findings detailed in Figs. 4, 5, and Supplementary Fig. S5, we immunostained the PLK1 protein in the FFPE specimens of 40 patients in the Test set. Seven of 13 (54%) cases in the low miR-886-3p expression group had strong expression of PLK1 (grade 2 and grade 3), whereas most patients in the high miR-886-3p expression group (10/13, 77%) had weak (grade 1) or no expression (Grade 0) of PLK1 (Supplementary Fig. S5A). The reverse correlation in the PLK1 level versus the miR-886-3p level was quite significant, \( P = 0.026 \) (Supplementary Fig. S5B).

miR-886-3p suppressed tumor growth and metastasis of the NCI-H446 xenograft in nude mice

To assess miR-886-3p’s effect on in vivo tumor repression, we made 2 pools of the NCI-H446 cell lines stably transfected with the pcDNA3.1-miR-886, or the control with an empty pcDNA3.1 vector (Mock), respectively. As expected, NCI-H446-miR-886-3p–transfected NCI-H446 cells expressed a much higher level of miR-886-3p (Fig. 6A), and had a decreased cell proliferation (Fig. 6B) and a reduced level of both PLK1 and TGF-\( \beta \)1 proteins (Fig. 6C; results not shown) than the Mock.

Both cell pools were implanted subcutaneously into 21 nude mice in each group, and tumor growth was monitored weekly. Tumors in the NCI-H446-miR-886-3p tumor grew significantly slower and resulted in smaller tumor mass [4443.84 mm\(^3\) (mean), 2788.51 mm\(^3\) (\(2\times\) SD) vs. 2454.64 (377.92 mm\(^3\); Fig. 6D and E], showing no sign of tumor invasion and metastasis. In contrast, 7 Mock mice suffered from tumor invasions to bones, one of which also had lung metastasis, and 9 suffered from tumor invasion into striated muscles, 2 of which had tumor thrombus (Fig. 6F).
Discussion

Lung cancer is one of the most deadly types of cancer. Its poor clinical outcome underscores a compelling need for efficient and novel measures for better early detection, prognosis prediction, and personalized therapies. SCLC accounts for 15% to 25% of all diagnosed lung cancers and attributes to 200,000 deaths each year worldwide (1). SCLC is notorious due to the rapid progression and frequent 2 years recurrence rate (41). There is mounting evidence for the critical involvement of deregulated miRs in both initiation and progression of human NSCLC (16, 17, 42), but the comparable information for SCLC is little (43, 44). In this study, we initiated a discovery phase exercise for the key miRs by surveying 924 known miRs expression in FFPE specimens of 42 SCLC cases and then engaged in systematic studies revealing mechanistic understanding and clinical relevance of the aberrant expression of miR-886-3p, one of the most promising candidates identified here. Loss of miR-886-3p expression was tightly associated with poor clinical outcome defined by OS, PFS, and DMFS (Fig. 1 and Supplementary Fig. S1).

Two major mechanisms are often considered instrumental to silencing of miR expression, epigenetic (e.g., aberrant DNA methylation and histone modifications) and genetic (e.g., loss of the genes by deletion). The genetic mechanisms may not be relevant here, as the most frequent deletions in SCLC are at 5q13-q21, rather than 5q31.1 where the miR-886-3p gene resides (45). The miR-886-3p promoter is located in a typical CpG island (Fig. 2A), and was hypermethylated in 3 low miR-886-3p-expressing SCLC cell lines (NCI-H446; Fig. 2; and NCI-H524, NCI-H209; Supplementary Fig. S3) and at a much higher frequency in the cancer tissues of the low expression group (Fig. 3 and Supplementary Table S3). We showed in this study that both the promoter hypermethylation and loss of miR-886-3p expression are indicative of the poor clinical outcome of SCLC.

The tumor suppressor status of the miR-886-3p was further supported by the observations that forced expression of miR-886-3p by Mimic transfection suppressed proliferation, migration, and invasion of cultured NCI-H446 cells (Fig. 4) and tumor growth, bone/muscle invasion, and lung metastasis in nude mice (Fig. 6). Among 3 bioinformatically predicted targets of miR-886-3p, 2 well-known active oncogenic genes, PLK1 and TGF-β1, are proven to be the true targets (Figs. 4 and 5). Significantly, the PLK1 gene expressed higher in the miR-886-3p low expressing/hypermethylated FFPE tissues and correlated well with the poor clinical outcome of SCLC (Supplementary Fig. S5). In summary, we have identified a novel nexus of DNA-methylated miR-886-3p/PLK1 and TGF-β1 that regulates the proliferation, migration, and invasion of SCLC cells in the cell culture system and tumor growth, invasion, and metastasis behaviors in vivo. It is therefore hypothesized that, in certain patients, SCLC is initiated when the miR-886-3p promoter gets hypermethylated, leading to loss of miR-886-3p transcription, which subsequently reverses the repression imposed by miR-886-3p on both PLK1 and TGF-β1 expression, to activate the relevant oncogenic pathways. Both activated pathways work in synergy, along with other fundamental changes to drive a cell to proliferate and develop to a more malignant stage of SCLC carcinogenesis.

It has been recently proposed that the miR-886-3p is a fragment of vault RNA (vtRNA), which was believed to play a role in cellular resistance to cancer therapeutic drugs (46, 47). However, the most recent findings maintained vtRNA is not a genuine precursor of miRNA-886-3p and miRNA-886-5p (48). Regardless of the arguments on the origin of miR-886-3p, this newly identified miR-886-3p-PLK1/TGF-β1 nexus regulating SCLC aggression suggests that both the loss of miR-886-3p expression and the hypermethylation of the miR-886 promoter along with PLK1 are likely the promising biomarkers for predicting the poor outcome of and targets for SCLC therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: J. Cao, Y. Song, N. Bi, J. Shen, J. Zhu, L. Wang, Q. Zhan
Development of methodology: J. Cao, N. Bi, L. Wang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Cao, N. Bi, J. Shen, W. Liu, J. Fan, Y. Shi, X. Zhang, N. Liu, Y. He, K. Ma, X. Luo, H. Deng, G. Sun
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Cao, Y. Song, N. Bi, J. Shen, W. Liu, H. Zhang, J. Zhu, L. Wang
Writing, review, and/or revision of the manuscript: N. Bi, J. Shen, J. Zhu, L. Wang, Q. Zhan
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Song, J. Shen, W. Liu, J. Fan, T. Tong, J. He, N. Lu, H. Zhang, L. Lu, J. Cheng
Study supervision: Y. Song, L. Wang, Q. Zhan

Grant support
This work was supported by The 973 National Key Fundamental Research Program of China (2006CB521801, 2010CB912800, 2009CB525606, 2009CB525607) and the National Natural Science Foundation of China (81021061, 3092110312).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 3, 2012; revised March 6, 2013; accepted March 14, 2013; published OnlineFirst April 16, 2013.
DNA Methylation Regulates miR-886-3p in SCLC

5. Kim H, Huang W, Jiang X, Pennicooke B, Park PJ, Johnson MD. Integrative genome analysis reveals an oncsmi/organome cluster regu-


13. Hurteau GJ, Carlson JA, Spivack SD, Brock GJ. Overexpression of the miRNomes in human liver and hepatocellular carcinoma reveals miR-


DNA Methylation-Mediated Repression of miR-886-3p Predicts Poor Outcome of Human Small Cell Lung Cancer

Jianzhong Cao, Yongmei Song, Nan Bi, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-12-3055

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2013/04/15/0008-5472.CAN-12-3055.DC1

Cited articles
This article cites 42 articles, 21 of which you can access for free at:
http://cancerres.aacrjournals.org/content/73/11/3326.full#ref-list-1

Citing articles
This article has been cited by 4 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/73/11/3326.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/73/11/3326.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.