DNA methylation mediated repression of miR-886-3p predicts poor outcome of human small cell lung cancer

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Abstract

Small-cell lung cancer (SCLC) is one of the most aggressive types of cancer, yet the pathological mechanisms underlying its devastating clinical outcome remain elusive. In this report, we surveyed 924 microRNA (miR) for their expressions in the formalin-fixed paraffin-embedded (FFPE) specimens from 42 SCLC patients, and found that the down-regulated 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 post-transcription 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 promoter are the promising indicators for poor outcome of as well as new therapeutic targets for SCLC.
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 15-25% 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.

MicroRNAs (miRs) are noncoding RNA of about 22 nucleotides in length and repress the gene expression at both mRNA translation and stability levels through pairing to complementary sites in the 3’-untranslated region (3’-UTR) of the target mRNA. A number of investigations have demonstrated that aberrant miR expression is closely related to the clinical progression in both solid organs and hematological system, including liver cancer [3], esophageal cancer [4], brain tumor [5], ovarian cancer [6], breast cancer [7], and leukemia [8]. Several miRs have been implicated in non-small cell lung carcinoma (NSCLC), including miR-1, miR-7, let-7, miR-17-92, miR-21, miR-200c and miR-221[9-15]. More recently, a global miR expression pattern determined by a microarray based omic analysis has been established and several NSCLC “miR signatures” have been proposed for the better molecular staging and classification of NSCLC [16-19]. The miRs overexpressed in human cancers, such as miR-21, miR-155 and miR-17-92 exert positive impact to carcinogenesis and are considered as oncogenic type of miRs [15, 20, 21]. Some miRs are downregulated in cancer cells, however, inhibit cell proliferation, invasion and migration in cell culture and tumor growth and metastasis. These miRs include miR-15a, miR-16-1, miR-199a/b-3p [22] and Let-7 [9, 23]. One of the mechanisms for de-regulated expression associated with cancer concerns the aberrant epigenetic changes of miR promoters, predominantly at the DNA methylation and histone modification levels.
For instance, both miR-124a and miR-127, two tumor suppressor miRs that negatively regulate proto-oncogene CDK6 and BCL6, are hypermethylated in tumor cells [25-27].

To identify the key miRs involved in SCLC carcinogenesis, we first surveyed 924 known miRs in formalin-fixed paraffin-embedded (FFPE) specimens of 42 patients and found a tight association of the loss of miR-886-3p expression with the poor clinical outcome of SCLC patients, such an association was further confirmed by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis of another 40 patients’ specimens. We further demonstrated 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 post-transcription level.

Methods

Clinical tissue samples and immunohistochemical analysis. The formalin-fixed paraffin-embedded (FFPE) specimens of 82 cases with histologically confirmed limited-stage SCLC, ECOG performance status of 0-1, 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.

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 samples 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 based on 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% - 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 scored was 0 were marked as (Grade 0), 1-2 marked as (Grade 1), 3-4 marked as (Grade 2), and 5-6 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 PEG solution precipitation method [29], and was labeled using the T4 RNA ligase labeling method described by Thomson et al [30]. Hybridization was carried to a microRNA microarray panel (CapitalBio, Beijing, China), which include probes in triplicate for 924 mature human and mouse miRNA sequences and eight short oligonucleotides that possessed no homology to any known RNA sequence as external controls. 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, Beijing, China)[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 Cluster 3.0 software before the unsupervised clustering with complete linkage were performed. Uncentered Pearson correlation to reveal the underlying structure of the miRNA expression [31]. Hazard ratios 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, GEO: GSM678225 - GSM678266).

Quantitative miRNA RT-PCR was performed 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, GTGCAGGGTGCGAGGT; miR-886-3p, CGCGGGTGCTTACTGACCCTT; miR-886-3p RT primer,
GTGATCCAGTGCGAGGTGACAGTGGACTTACTGAACG; miR-886-3p forward primer, CACGCGGTGCTTACTGAC. With U6 forward primer, CTCGCTTCCGCAGCACA; and U6 reverse primer, AACGCTTCAGAATTGCGT qRT-PCR was performed on an ABI Prizm 7300 Sequence Detection System using LightCycler DNA Master SYBR Green I mix (Roche Molecular Biochemicals, Mannheim, Germany) 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 et al, 2005) and normalized with the level of the internal control, U6, plotted for inter-cellular comparison.

**Cell culture, transfection and analysis.** Four small cell cancer cell lines, NCI-H69 (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, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY). The miR-886-3p mimic (Mimic) and unrelated mimic control (miR-NC) were purchased from Genechem (Shanghai, China) and RiboBio (Guangzhou, China), respectively. The mimic transfection using Lipofectamine2000 (Invitrogen, Carlsbad, CA) was performed as previously described [32].

pGL3-Plk1-3’UTR and pGL3-TGF-β1-3’UTR were constructed by inserting the 3’UTR regions (Table S1) of Plk1 and TGF-β1 into the XbaI sites of pGL3-control plasmid. PGL3-Plk1-3’UTRmut harbors a substitution of eight nucleotides within the core binding region of the PLK1 3’UTR and the pGL3-TGF-β1-3’UTRmut carried a substitution of seven nucleotides in the binding sites of TGF-β1. NCI-H446 cells were transfected with 50 ng of each reporter construct/10 ng pRenilla (the control for transfection) and 100 nM 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, $2 \times 10^4$ cells were seeded into six-well plates 24h after transfection. Cells were harvested daily and counted in a hemocytometer for 4 days in triplicate. The Costar Transwell 8 μm inserts were coated with 50 μg reduced serum Matrigel (BD Biosciences Franklin Lakes, NJ) for the invasion assay according to the manufacturer's instruction. RPMI-1640 was used to resuspend the cells at 48h post-transfection. Migration assays were performed in the same manner excluding the Matrigel. After 12h, non-invading cells, and media were removed with a cotton swab. The underside of the inserts was stained with 0.5% crystal violet (Sigma). Ten random fields for each insert were counted. Inserts were conducted in triplicate in three separate experiments [33].

The pcDNA3.1-miR-886-3p expression vector was made by inserting the coding region of the miR-886 gene (chr5: nt 135443876-135444376) into pcDNA3.1 vector. pEGFP-N1-PLK1 and pEGFP-N1-TGF-β1 expression vectors were made by cloning human cDNA of PLK1 (NM_005030.3, nt 51-1862, 1827bp) and TGF-β1(NM_000660.4, nt 884-2053, 1182bp) into pEGFP-N1 vectors, respectively. To establish the miR-886-3p expressing NCI-H446 cell line, $4 \times 10^5$ cells were transfected with pcDNA3.1-miR-886-3p and selected with G418 (400μg/ml) for one week and pooled for future 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 two non-cancerous patients. A bisulphite conversion was carried out with a modified form of Hayatsu’s protocol [35]. The CpG Island Searcher Program [36] was used to determine if any CpG island existed within the miR-886 locus (one kilobases upstream and downstream of the miR-886 coding region) [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 (Table S2).

**5-Aza-2'-deoxycytidine and TSA treatment of the NCI-H446 cell line.**
NCI-H446 cells were treated with 1.5 μM 5-Aza-2′-deoxycytidine (Sigma A3656) for 2 days or 4 days. Twenty-four hours prior to harvest, 0.5μM 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 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 upper back and monitored for tumor volumes, overall health and total body weight. The size of the tumor was determined by caliper measurement of the subcutaneous tumor mass. Tumor volume were calculated according to the formula 4/3πr1×r2²(r1 > r2). Each experimental group contained 7 mice and the experiments were repeated three times [33].

Statistical analysis. Student’s t-test or χ²-test was used to calculate the difference in patient clinical characteristics. Overall survival (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, while 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 upper one-third and low expression was classified as the lower one-third in each sample group. Survival curves were estimated with the Kaplan-Meier method and compared with the log-rank test. Cox proportional hazard regression was then used to identify miRNAs that were correlated with the overall survival (OS). χ²-test was used to compare PLK levels in different miR-886-3P expression groups. All P values were two sided, and a P value of less than 0.05 was
considered to be statistically significant. All the analyses were performed using the SPSS software package (version 11.5, SPSS Inc., Chicago, IL).

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 formalin-fixed paraffin-embedded (FFPE) specimens from 42 SCLC patients (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), two 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 (concordance Coefficients: 0.85, P=0.002). Patients were equally divided to three groups (14 cases per group): high (upper 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 overall survival than the high expression group: the five-year survival rate in the low was 27.0%, versus 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 five-year OS rate was 15.0% in the low versus 77.0% in the high expression group, P<0.0001, Fig. 1c).

The overall survival (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 two prognostics measurements: progression free survival (PFS) and distance metastasis free survival (DMFS) were also significantly worse in the low than the in the high
expression group (P=0.001 in PFS and P=0.0005 in DMFS, respectively, Fig. 1e-f), Fig. S1a-e). Therefore, the down-regulation of miR-886-3p appeared to contribute to tumor progression of 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 bisulfite sequencing (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 two non-cancerous lung disease patients (Fig. 2b). While 93.8% and 100% of CpGs were unmethylated in Normal 1 and 2, only 8.1% of 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 methylation specific PCR (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), while 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 methyl-transferase inhibitor, 5-Aza-2'-deoxycytidine and the DNA methylation status of the miR-886-3p promoter was determined by BSP (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, Fig. S2b and c). 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, while the level of other three CpG island-free miR genes: miR-17, miR-31 and miR-150, remained unchanged (Fig. S2d). Furthermore, both PLK1 and TGF-β1 proteins, the two primary targets of miR-886-3p, were down-regulated to about 50% of the level of the Mock treated (Fig. S2e).
A compatible analysis was extended to three additional SCLC cell lines: NCI-H209, NCI-H524 and NCI-H69 cells. The miR-886-3p promoter was almost fully methylated in the former two (11.4 and 13% CpGs unmethylated), but essentially unmethylated in the NCI-H69 cell line. (78.1% CpGs unmethylated, Fig. S3a and b). 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 (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 33 patient’s samples with known miR-886-3p expression levels: 17 cases in the low and 16 cases in the high expression groups (Fig 3a-c and Table S3). The miR-886 promoter was methylated in 9, unmethylated in 1 and partially methylated in 7 low expression cases, respectively. Significantly, the unmethylated, partially methylated and methylated miR-886-3p promoter was seen in 10, 5 and 1 high expression cases, respectively. The DNA methylated status of the promoter correlated well with the overall survival of SCLC patients (P=0.019) (Fig 3c), suggesting the predicative value of the hypermethylation of the miR-886 promoter for the poor clinical outcome of SCLC.

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 status for miR-886-3p. Taking both confidence score and their known roles in carcinogenesis into consideration, mRNA of the following three candidates, PLK1, TGF-β1, and MYBBP1A, predicted by TargetScan4.0 software were qRT-PCR 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 mRNA of MYBBP1A mRNA (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 pl-G3-Plk1-3’UTR, pl-G3-Plk1-3’UTRmut, pl-G3- TGF-β1-3’UTR, and pl-G3-TGF-β 1-3’UTRmut constructs, respectively (Fig. 5a and b). Each construct was co-transfected with Mimic or miR-NC and luciferase activity was quantified and compared. As expected, the luciferase activities of both pl-G3-Plk1-3’UTR and pl-G3- 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, migration /invasion created by Mimic transfection were reversed by transfection of both expression constructs (Fig. S4a-c).

To confirm the clinical relevance of the findings detailed in Fig. 4, 5 and Fig. S5, we immunostained the PLK1 protein in the FFPE specimens of 40 patients in the Test set. 7/13 (54%) cases in the low miR-886-3p expression group had strong expression of PLK1 (Grade 2 and Grade 3), while most patients in the high miR-886-3p expression group (10/13, 77%) had weak (Grade 1) or no expression (Grade 0) of PLK1 (Fig S5a). The reverse correlation in the PLK1 level versus the miR-886-3p level was quite significant, P=0.026 (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 two 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-β1 proteins (Fig. 6c and Results not shown) than the Mock.

Both cell pools were implanted subcutaneously into 21 nude mice in each groups, 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$^3$ (2X SD) versus 24546.44 , 3777.92 mm$^3$) (Fig. 6d and 6e), 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 15-25% of all diagnosed lung cancers and attributes to approximate 200,000 deaths each year worldwide [1]. SCLC is notorious due to the rapid progression and frequent two years recurrence rate [41]. There is mounting evidence for the critical involvement of de-regulated 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 overall survival (OS), progression free survival (PFS) and
distance metastasis free survival (DMFS) (Fig. 1 and 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 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 three low miR-886-3p expressing SCLC cell lines (NCI-H446, Fig 2, and NCI-H524, NCI-H209, Fig.S3) and at a much higher frequency in the cancer tissues of the low expression group (Fig. 3 and Table S3). We demonstrated 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 three bioinformatically predicted targets of miR-886-3p, two well-known active oncogenic genes: PLK1 and TGF-β1 are proven to be the true targets (Fig. 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 (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 reverse 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, nevertheless, 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.

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**References**


Table 1 Clinical characteristics of the LD-SCLC patients according to miR-886-3p levels

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<td>Female (%)</td>
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LD, limited stage disease; SCLC, small cell lung cancer

* Details of ECOG Performance Status are listed in the supplementary Table S4.
Fig. Legends

Fig. 1 Kaplan-Meier analyses of overall survival (OS), progression free survival (PFS) and distant metastasis free survival (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 (Testing group). The SCLC patients were equally divided into high (Upper one third), medium (middle one third) and low (bottom one third) groups. The OS, PFS and DMFS were analyzed in the Training, Testing and Full group. (a) Summary table. (b) OS in Training group. (c) OS in Testing group. (d) OS for patients in both Training and Test groups (Full group). (e) PFS and (f) DMFS in full group.

Fig. 2 DNA methylation status of the miR-886-3p promoter. (a) The miR-886-3p gene. The vertical bars: the CpG dinucleotide in the miR-886 locus and arrows: the coding region of 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 to -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 for the methylated allele and UT for the unmethylated alleles) 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 refers methylated state, and U refers unmethylated state. +: the positive controls for the methylated and unmethylated state, respectively. -: the no template control.

Fig. 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 and U, 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 SCLC patients in this analysis (P=0.001). (e) Kaplan-Meier curve of estimated OS for SCLC patients with different methylated states of miR-886-3p gene (P=0.019).

**Fig. 4** miR-886-3p’s suppressive effects on cell proliferation and invasion of NCI-H446 cells. (a) MiRs level in the miR-886-3p mimic (Mimic) versus the non-related mimic (miR-NC) transfected NCI-H446 cells by qRT-PCR and normalized with U6. (b) Mimic’s effect on cell proliferation. (c) Mimic’s effect on cell invasion and migration. (d) The data in (c) was calculated and plotted. (e) The Mimic’s effect on the mRNA level of PLK1, TGF-β1, and MYBBP1A. (f) Mimic’s effect on the PLK1 and TGF-β1 level by Western analysis, the relative level (the Mimic transfected versus miR-NC) was quantified and indicated.

**Fig. 5** miR-886-3p’ repressing activities targeted the 3’-untranslated regions (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 firefly 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

**Fig. 6** miR-886-3p’s effect on *in vivo* tumor growth and metastasis. (a) The pooled NCI-H446 cells stably transfected with the miR-886-3p expression vector: pcDNA3.0-miR-886-3p (NCI-H446-miR-886-3p) or empty vectors (NCI-H446-control) were established and the level of miR-886-3p were qRT-PCR analyzed. (b) Both PLK1 and TGF-β1 proteins were quantified by Western blotting analysis. (c) Cell proliferation profile of NCI-H446-miR-886-3p and NCI-H446-control. (d) Growth of both NCI-H446-miR-886-3p and NCI-H446-control cells induced tumor were in nude mice was monitored weekly. (e)
The representative tumor mass. (f) The cytological features of representative tumors from the NCI-H446-control cells by HE analysis. (a’) Lung metastasis (n=1). (b’) Striated muscle invasion (n=9). (c’) Bone invasion (n=7). (d’) Tumor thrombus (n=2). Original magnification, ×100.
**Figure 1**

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<td>28</td>
<td>27</td>
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</table>

- **b)** Training group-miR-886-3p
  - High expression (N=14)
  - Low expression (N=14)
  - OS (%) vs Months
  - $P=0.045$

- **c)** Test group-miR-886-3p
  - High expression (N=13)
  - Low expression (N=13)
  - OS (%) vs Months
  - $P=0.0001$

- **d)** Full group-miR-886-3p
  - High expression (N=27)
  - Low expression (N=27)
  - OS (%) vs Months
  - $P<0.0001$

- **e)** Full group-miR-886-3p
  - High expression (N=27)
  - Low expression (N=27)
  - PFS (%) vs Months
  - $P=0.001$

- **f)** Full group-miR-886-3p
  - High expression (N=27)
  - Low expression (N=27)
  - DMFS (%) vs Months
  - $P=0.0005$
Figure 2

(a) Schematic representation of CpG Islands with miR-886-3p, MSP region (126-131bp), and BSP region (196bp).

(b) DNA sequence around the miR-886-3p BSP region.

(c) miR-886-3p BSP region with methylation status for Normal 1, Normal 2, and NCI-H446.

(d) Bar graph showing unmethylated CpG (%) with values 93.8, 100, 96.9, and 8.1.

(e) Gel electrophoresis showing bands at 126 bp (M) and 131 bp (U) for NCI-H446, Normal 1, and Normal 2.
Figure 3

a

![Marker 8 9 10 11 17 18 with 126bp (M) and 131bp (U) markers]

b

<table>
<thead>
<tr>
<th></th>
<th>miR-886-3p level</th>
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<td>Partially</td>
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P=0.001

c

![Kaplan-Meier curves for survival with Unmethylated, Partially methylated, and Methylated groups with P=0.019]
Figure 4

(a) Relative miRNA levels for miR-886-3p, miR-31, and miR-17.

(b) Cell number (10^4) over time for miR-NC and Mimic.

(c) Images of migration and invasion for miR-NC and Mimic.

(d) Invasion or migration levels for miR-NC and Mimic.

(e) Relative levels of mRNA for PLK1, TGF-β1, and MYBBP-1A.

(f) Western blot images for PLK1, TGF-β1, and Actin.
Figure 5

(a) miR-886-3p

miR-886-3p

Plk1

3’_uucccaGUCAUUCGUGGGCGG 5’

ccatacTGGTTGCTCCCGCG

5’_ggctGTATTTAAGGACACCGTG

miR-886-3p

TGF-β1

3’_uuccCAGUCAUUC—GUGGGCGG 5’

ccatacTGGTTGCTCCCGCG

ccatacACCTACGAGGCGG

(b) Luciferase

Plk1-3’UTR

pLG3-Plk1-3’UTR

cccatacTGGTTGCTCCCGCG

pLG3-Plk1-3’UTRmut

cccatacACCTACGAGGCGG

Luciferase

TGF-β1-3’UTR

pLG3-TGFβ1-3’UTR

ggctGTATTTAAGGACACCGTG

pLG3-TGFβ1-3’UTRmut

ggctCAATTATTGAGGCTGGCTG

(c)

(d)

1.3

Luciferase activity

1.4

0.5

(pGL3-Control + + + - - - - - -)

pGL3-Plk1-UTR - - - + + + + + +

pGL3-Plk1-UTRmut - - - - - + + + +

miR-NC - + - - + - - + -

miR-886-3p - - - + - - + + +

1.1

Luciferase activity

1.4

0.4

(pGL3-Control + + + - - - - - -)

pGL3-TGF-β1-UTR - - - + + + + + +

pGL3-TGF-β1-UTRmut - - - - - + + + +

miR-NC - + - - + - - + -

miR-886-3p - - - + - - + + +

1.0

0.6

0.4

0.2

0
Figure 6

a. miR-886-3p levels (%) in NCI-H446-control and NCI-H446-miR-886-3p.

b. Western blots of PLK1, TGF-β1, and Actin in NCI-H446-Control and NCI-H446-miR-886-3p.

c. Cell number (x10^4) over time for NCI-H446-Control and NCI-H446-miR-886-3p.

d. Tumor volume (mm^3) over time for NCI-H446-Control and NCI-H446-miR-886-3p.

e. Images of NCI-H446-control and NCI-H446-miR-886-3p.

f. Histological images showing:
   - Lung metastasis (n=1)
   - Striated muscle invasion (n=9)
   - Bone invasion (n=7)
   - Tumor thrombus (n=2)
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.

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