miR-186 Downregulation Correlates with Poor Survival in Lung Adenocarcinoma, Where It Interferes with Cell-Cycle Regulation

Junchao Cai1,3, Jueheng Wu1,3, Huizhong Zhang4, Lishan Fang1,3, Yongbo Huang1,3, Yi Yang2,3, Xun Zhu1,3, Rong Li1,3, and Mengfeng Li1,3

Abstract

Deeper mechanistic understanding of lung adenocarcinoma (non–small cell lung carcinoma, or NSCLC), a leading cause of cancer-related deaths overall, may lead to more effective therapeutic strategies. In analyzing NSCLC clinical specimens and cell lines, we discovered a uniform decrease in miR-186 (MIR186) expression in comparison with normal lung tissue or epithelial cell lines. miR-186 expression correlated with patient survival, with median overall survival time of 63.0 or 21.5 months in cases exhibiting high or low levels of miR-186, respectively. Enforced overexpression of miR-186 in NSCLC cells inhibited proliferation by inducing G1–S checkpoint arrest. Conversely, RNA interference–mediated silencing miR-186 expression promoted cell-cycle progression and accelerated the proliferation of NSCLC cells. Cyclin D1 (CCND1), cyclin-dependent kinase (CDK)2, and CDK6 were each directly targeted for inhibition by miR-186 and restoring their expression reversed miR-186–mediated inhibition of cell-cycle progression. The inverse relationship between expression of miR-186 and its targets was confirmed in NSCLC tumor xenografts and clinical specimens. Taken together, our findings established a tumor-suppressive role for miR-186 in the progression of NSCLC.

Introduction

Lung cancer is well recognized as a leading cause of cancer-related mortality and morbidity worldwide, with more than 1.23 million cases newly diagnosed each year average for the past 2 decades (1, 2). Nearly 1 in 5 cancer-related deaths suffer from lung cancer, and the 5-year survival rate for the disease is lower than 15% (2). Non–small cell lung carcinoma (NSCLC), which includes squamous cell carcinoma, adenocarcinoma, adenosquamous cell carcinoma, large cell carcinoma, and several other histologic types, and is responsible for almost 80% of lung cancer–related deaths, represents the most prevalent histologic subtype of the tumor type (3). Currently available treatment strategies for NSCLC, including surgery, radiotherapy, and chemotherapy, however, remain generally unsuccessful (4). For example, the 5-year survival rate for patients with stage IV NSCLC is only approximately 1%, with the median survival time of 7 months (5). Despite incremental advancements in molecular therapeutics for the treatment of NSCLC, such as epithelial growth factor receptor–targeting therapies, patient survival has not been improved significantly, and the disease remains to be a long-term challenge with a dismal prognosis (3–6). Identification of molecular events key to the carcinogenesis and tumor progression will facilitate development of new therapeutic targets and anti-lung cancer strategies.

As characterized, rapid and unrestrained cell proliferation is a fundamental basis of the malignant phenotype of cancer, not only for the development and growth of primary tumor, but also for the colonization of metastatic tumor cells in their target organs. Disturbed surveillance of cell-cycle progression, such as cellular evasion of multiple cell-cycle checkpoints, which can be driven by abnormal activation of cell-cycle regulators such as cyclins and cyclin-dependent kinases (CDK), represents a hallmark of tumor development and progression (7, 8). For example, cyclin D1, which can act to trigger early G1 entry from quiescence and facilitate G1 progression by cooperating with CDK4/6, has been shown to be a key driver factor of malignant transformation of NSCLC (9, 10). CDK6 and CDK2, activated by binding to cyclin D and cyclin E, respectively, during the G1–S cell-cycle transition, are critical regulators in sequential modifications of retinoblastoma (Rb) pathway (11, 12), disruption of which exhibits strong cell-proliferative activity and has been shown as a mechanism for the pathogenesis of lung cancer (13). Aberrant activation of the cyclin/CDK complexes can be partly ascribed to the loss or
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inactivation of endogenous CDK inhibitors, such as p15\textsuperscript{ink4b}, p16\textsuperscript{ink4a}, p21\textsuperscript{Cip1/Waf1}, and p27\textsuperscript{Kip1} (14). In addition, overexpression of distinct cyclins and CDKs also play crucial roles in the development and progression of NSCLC and have been shown to override the inhibitory effect of the above negative regulators (7, 15). The underlying mechanism that modulates the abundance of the cyclins and CDKs in NSCLC, however, remains largely unknown.

miRNAs function as important posttranscriptional repres- sors that selectively bind to target mRNAs, and thus modulate their cellular abundance or expression (16). The characteristics that a single miRNA can target multiple mRNAs enables this class of modulatory molecules to synergistically suppress various regulators that are cooperatively involved in cell-cycle progression, and identification of such a miRNA might provide new clue for the development of targeting therapy against lung carcinogenesis. On the other hand, it has been widely recog- nized that cancer-associated human miRNA genes are prefer- entially located at chromosomal fragile sites. For example, numerous miRNAs reported to be downregulated in NSCLC (17–19), including let-7g, miR-9-1, miR-34a, miR-101-1, miR-198, miR-29b/c, miR-26a/b, and miR-30a/c, are located at chromosome region 1p31.1, an area displaying high frequen- cies of allelic loss and microsatellite instability in NSCLC (20, 21). In our preliminary studies, 48 miRNAs were identified to be located at this region when analyzed using the University of California Santa Cruz (UCSC) genome browser. Of note, among theses miRNAs, miR-186 was of particular interest as our infor- matic analysis predicted potential binding sites for miR-186 in the 3’-untranslated regions (3’-UTR) of cyclin D1, CDK2, and CDK6, 3 key regulators of the cell cycle. In this report, we experimentally show that miR-186 indeed simultaneously targets cyclin D1, CDK2, and CDK6, leading to cell-cycle arrest. Importantly, miR-186 expression in NSCLC cells is abrogated and markedly reduced, resulting in uncontrolled cell-cycle pro- gression and consequently increased cell proliferation. More- over, miR-186 downregulation correlates with poor prognosis of patients with NSCLC, suggesting that miR-186 might serve as a tumor-suppressor miRNA in the development and progression of NSCLC.

Materials and Methods

Cell culture

Primary normal lung epithelial cells (NLEC) were obtained according to a previous report (22). In brief, surgically resected specimens of normal lung tissue were promptly removed and transported aseptically in Hank’s solution (Invitrogen) supplemented with 100 U/ml penicillin, 100 μg/mL streptomycin (Invitrogen), and 5 μg/mL gentamycin (Invitrogen). The tissue specimens were then incubated with 1.5 U/ml dispase (Roche Molecular Biochemicals) at 4°C overnight, and the epithelium was dissected and incubated with trypsin (Invitrogen). The reaction was stopped with soybean trypsin inhibitor (Sigma) and centrifuged, followed by resuspension in keratinocyte-serum-free medium (KSF) supplemented with 40 μg/mL bovine pituitary extract, 1.0 ng/mL EGF, 100 U/mL penicillin, 100 μg/mL streptomycin, 5 μg/mL gentamycin, and 100 U/mL nystatin (Invitrogen). The BEAS-2B immortalized human bronchial epithelial cell line (Shanghai Institutes of Biological Sciences, Shanghai, China) was cultured in LHC-9 medium as instructed by the provider. Lung cancer cell lines, including HLAMP, A549, 95D, Calu-3, SK-MES-1, PAa, NCI-H460, NCI-H292, NCI-H596, NCI-H1299, NCI-H1650, and NCI-H1975, were obtained from cell banks of Shanghai Institutes of Biological Sciences or Fu Erbo Biotechnology Co., Ltd., and maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% FBS (HyClone) and 1% penicillin/streptomycin (Invitrogen). All cell lines were authenticated by short tandem repeat (STR) fingerprinting at Medicine Laboratory of Forensic Medicine Department of Sun Yat-Sen University (Guangzhou, China).

Tissue specimens

Clinical tissue samples used in this study were histopath- ologically and clinically diagnosed at the Sun Yat-Sen University Cancer Center (Guangzhou, China) from 2000 to 2004. The histologic characterization and clinicopathologic staging of the samples were determined according to the current International Union Against Cancer (UICC) Tumor–Node–Metas- tasis (TNM) classification. For the use of these clinical materi- als for research purposes, prior patients’ consents and approval from the Institutional Research Ethics Committee were obtained. Clinical information of the samples is presented in Supplementary Table S1.

RNA extraction and real-time quantitative PCR

Total miRNA of cultured cells, surgically resected fresh NSCLC tissues, and paraffin-embedded, archived clinical NSCLC specimens were extracted using the mirVana miRNA Isolation Kit (Ambion) and RecoverAll Total Nucleic Acid Isolation Kit (Ambion) according to the manufacturer’s instruction. cDNA was synthesized with 5 ng of total RNA using the TaqMan miRNA Reverse Transcription Kit (Applied Biosystems), and the expression levels of miR-186 were quan- tified with the miRNA-specific TaqMan MiRNA Assay Kit (Applied Biosystems).

Western blotting

Western blotting was conducted according to a standard method previously described (23), using anti-CDK2, anti-CDK6, or anti-cyclin D1 antibodies (BD PharMingen). Blotted membranes were stripped and reblotted with an anti-glycer- aldehyde-3-phosphate dehydrogenase (GAPDH) rabbit mono- clonal antibody (Sigma) as a loading control. Relative protein levels were quantified by scanning densitometry, and the relative gray value of protein was calculated as band intensity of protein of interest/ band intensity of loading control.

RNA immunoprecipitation

Co-immunoprecipitation (Co-IP) of microRNA ribonucleo- protein complex (miRNP) with anti-Ago1 (Abcam) or immu- noglobulin G (IgG; Sigma) was conducted as previously described (24). RNA coimmunoprecipitated with anti-Ago1 or IgG antibodies was extracted using TRIzol LS (Invitrogen) as described in a previous publication (25). Total RNA was sub- jected to DNA digestion as described earlier.
Xenografted tumor model in vivo and immunohistochemistry

Female BALB/c-nu mice (5–6 weeks of age, 18–20 g) were purchased and housed in barrier facilities on a 12 hours light/dark cycle. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Sun Yat-Sen University. For bioluminescent imaging assay, indicated cells (8 × 10^6, suspended in 100 μL sterile PBS, A549-luc-Vector left and A549-luc-miR-186 right) were injected subcutaneously into the dorsal thigh of female BALB/C nude mice (n = 5). Bioluminescent imaging was conducted using Xenogen IVIS Spectrum (Caliper Life Sciences). Fifteen minutes prior imaging, mice were injected intraperitoneally with 150 mg/kg luciferin. Following general anesthesia, images were taken and analyzed with Spectrum Living Image 4.0 software (Caliper Life Sciences). Tumor growth was monitored weekly by in vivo imaging and photon radiance measurement. After 5 weeks, mice were anesthetized and sacrificed, and tumors were removed, weighed, and sectioned (5 μm in thickness), followed by immunohistochemical (IHC) analysis. After deparaffinization, sections were immunohistochemically analyzed using anti-Ki-67 (Sigma), anti-CDK2, anti-CDK6, and anti-cyclin D1 antibodies (BD Pharmingen), respectively. The resultant immunostaining images were captured using the AxioVision Rel.4.6 computerized image analysis system (Carl Zeiss).

Statistical analysis

A cohort of 140 patients with NSCLC was divided into 2 groups based on miR-186 expression level, namely, the low-miR-186 expression group (less than the median value) and the high-miR-186 expression group (more than the median value), for clinical survival analysis. All statistical analyses were carried out using the SPSS 13.0 statistical software package. Comparisons between groups for statistical significance were conducted with a 2-tailed paired Student t test. The χ² test was used to analyze the relationship between miR-186 expression and clinicopathologic characteristics. Survival curve was plotted using the Kaplan–Meier method and compared by the log-rank test. P < 0.05 was considered statistically significant in all cases.

Results

miR-186 was downregulated in NSCLC

To investigate the potential significance of miR-186 in the development and progression of NSCLC, we first examined the expression of the miRNA in NSCLC cell lines and tissues. Real-time PCR analysis showed that miR-186 was ubiquitously expressed at lower levels in a panel of 12 human lung cancer cell lines than in cultured primary NLEC and immortalized human bronchial epithelial cell line BEAS-2B (Fig. 1A). In parallel, as shown in Fig. 1B, miR-186 expression was found to be markedly decreased in all 8 collected NSCLC tumor lesions as compared with that in paired adjacent noncancerous lung tissues. These data strongly suggested that miR-186 expression was significantly suppressed in NSCLC.

miR-186 downregulation correlated with NSCLC clinicopathologic characteristics and the overall survival of NSCLC patients

The observed downregulated expression of miR-186 in lung cancer prompted us to further investigate the clinical relevance of miR-186 in the progression of NSCLC. We therefore extended our miR-186 quantification experiment to a cohort of 140 archived paraffin-embedded specimens of NSCLC (Supplementary Table S1), and our data showed that miR-186 was differentially detectable in all tumor specimens surgically resected from patients with NSCLC. As shown in Supplementary Table S2, the level of miR-186 expression closely correlated with NSCLC clinical staging (P < 0.001) and TNM classification (P = 0.001, P < 0.001, and P = 0.014, respectively). Furthermore, Kaplan–Meier analysis using the log-rank test was conducted and the result showed that patients with high miR-186 expression in their lung tumors had a longer median survival time of 63.0 months than those with low miR-186 expression, whose median survival time was 21.5 months (P = 0.012; Fig. 1C). Moreover, we found that patients with tumors exhibiting low miR-186 expression exhibited significantly shorter overall survival than those with high expression of miR-186 in either the stage I–II subgroup (n = 80; P = 0.006, log-rank; Fig. 1D) or the stage III–IV subgroup (n = 60; P = 0.025, log-rank; Fig. 1E). Thus, low miR-186 expression seemed to be a risk factor predicting poor survival, suggesting that decreased expression of miR-186 likely contributes to NSCLC pathogenesis and might represent a prognostic biomarker for the disease.

Overexpression of miR-186 suppressed cell proliferation and caused cell-cycle arrest in NSCLC cells

In the attempt to understand the biologic function of miR-186, miR-186 was stably transduced into the A549 and the SK-MES-1 NSCLC cell lines, respectively, to generate A549-miR-186 and SK-MES-1-miR-186 lines (Supplementary Fig. S1). As shown in Fig. 2A, ectopic miR-186 expression slowed down the propagation of the NSCLC cells, as analyzed using MTT assay. Furthermore, the capabilities of both cell lines to form colonies were robustly compromised by miR-186 transduction as compared with corresponding control cells (Fig. 2B). Moreover, soft agar assay revealed that miR-186 overexpression dramatically attenuated anchorage-independent growth abilities of the NSCLC cells, as evidenced by the number as well as the size of formed colonies measured, with miR-186–overexpressing cells generating far less and smaller colonies than vector-control cells (Fig. 2C). Taken together, these results suggested that overexpression of miR-186 suppressed the ability of NSCLC to proliferate in vitro.

To dissect the biologic events accompanying the alterations of cell proliferation caused by miR-186, fluorescence-activated cell sorting (FACS) was applied to analyze changes of DNA content throughout various phases of the cell cycle. As shown in Fig. 2D, both miR-186–overexpressing A549 and SK-MES-1 cells displayed a significant increase in the percentages of cells in G1-phase but decreased proportions of S-phase cells. Furthermore, BrdUrd incorporation assay confirmed that A549-miR-186 and SK-MES-1-miR-186 contained less BrdUrd-positive cells with newly synthesized DNA, 25.4% and
9.8%, respectively, than those in the control cell populations (41.8% and 20.0%, respectively, for A549-vector and SK-MES-1-vector cells; Fig. 2E). Thus, our data suggested that miR-186 interfered with the G1–S transition of cell-cycle progression and consequently abrogated the proliferation of NSCLC cells.

Antagonizing miR-186 accelerated the proliferation of NSCLC cells

To understand the role of endogenous miR-186 in the modulation of cell proliferation, anti-miR-186 oligonucleotides were synthesized and used as antagonists to silence endogenous miR-186 expression (Supplementary Fig. S2). As shown in Fig. 3A, antagonizing miR-186 in A549 and SK-MES-1 NSCLC cells drastically accelerated their proliferation as compared with their corresponding vector-control (NC) cells. Furthermore, clonogenic formation and anchorage-independent growth assays revealed that after transfected with miR-186 antagonists, both A549 and SK-MES-1 cells formed more and larger-sized colonies (Fig. 3B and C). In parallel, antagonizing miR-186 significantly decreased the proportion of G1-phase NSCLC cells but elevated the percentages of cells in the S-phase (Fig. 3D). Immunofluorescent staining for BrdUrd incorporation further revealed increased DNA synthesis by the miR-186 antagonists, showing 52.3% and 36.2% BrdUrd-positive cells in the A549 and the SK-MES-1 lines, respectively, as compared with 39.6% and 20.0% for the corresponding vector-control cells (Fig. 3E). Collectively, our antagonism experiments indicated that endogenous miR-186 might function as a cell-cycle suppressor capable of abrogating the entry of NSCLC cells to the S-phase and thus suppressing the progression of cell proliferation.

miR-186 targeted cell-cycle regulators cyclin D1, CDK2, and CDK6

To understand the mechanism underlying the robust suppressive effect of miR-186 on the proliferation of NSCLC cells, by using TargetScan, a bioinformatic tool for miRNA target screening, we found that 3 central cell-cycle promoting genes,
Figure 2. Overexpression of miR-186 suppressed NSCLC cell proliferation through abrogating the G1–S transition. A, MTT assays revealed cell growth curves of indicated cells. B, representative micrographs (left) and relative quantification (right) of crystal violet–stained cell colonies analyzed by clongenic formation. C, representative micrographs (left) and relative quantification (right) of colonies that were in diameter between 25 and 50 μm for A549 cells or between 10 and 20 μm for SK-MES-1 cells, counted in anchorage-independent growth ability assay. D, flow-cytometric determination of proportion of indicated cells in distinct cell-cycle phases. E, representative micrographs (left) and quantification (right) of BrdUrd-incorporated cells in indicated engineered cell lines. Error bars represent the means of 3 independent experiments. *P < 0.05; **P < 0.01. DAPI, 4',6-diamidino-2-phenylindole.
namely, *cyclin D1*, CDK2 and CDK6, were tentative targets of miR-186 (Fig. 4A). As shown in Fig. 4B, Western blotting confirmed that the protein levels of *cyclin D1*, CDK2, and CDK6 were indeed reduced drastically in miR-186–transduced cells but pronouncedly elevated in miR-186–silenced cells, as compared with those in the corresponding control cells.

**Figure 3.** Antagonism of miR-186 accelerated proliferation of NSCLC cells and promoted cell-cycle progression. A, MTT assays revealed cell growth curves of indicated cells. B, representative micrographs (left) and relative quantification (right) of crystal violet–stained cell colonies analyzed by colony formation assay. C, representative micrographs (left) and relative quantification (right) of colonies sized in diameter between 50 and 100 μm for A549 cells or between 20 and 50 μm for SK-MES-1 cells were determined. D, flow-cytometric analysis shows the proportion of indicated cells after 48 hours transfection with anti-miR or NC oligonucleotides. E, representative micrographs (left) and quantification of BrdUrd-incorporated cells in indicated cells. Error bars represent the means of 3 independent experiments. *, *P < 0.05; **, *P < 0.01.
Figure 4. miR-186 directly targeted cyclin D1, CDK2, and CDK6 cell-cycle regulators. A, predicted binding of miR-186 to 3’-UTRs of cyclin D1, CDK2, and CDK6. B, Western blotting of cyclin D1, CDK2, and CDK6 expression in indicated cells. GAPDH was used as a loading control. C, RIP analysis revealed recruitment of cyclin D1, CDK2, and CDK6 mRNAs to miRNP complex following immunoprecipitation against Ago1. IgG immunoprecipitation was used as a negative control. D, luciferase activity of pGL3-cyclin D1-3’-UTR, pGL3-CDK2-3’-UTR, or pGL3-CDK6-3’-UTR reporter in indicated cells cotransfected with indicated oligonucleotides. E, the effect of restoring ORFs (without 3’-UTRs) of cyclin D1, CDK2, or CDK6 on proportions of indicated cells in distinct cell-cycle phases as determined by flow-cytometric analysis. For C to E, data are presented as mean ± SD from 3 independent experiments. *P < 0.05; **P < 0.01.
despite that miR-186 had no impact on the mRNA levels of these putative targets (data not shown). In consistence with these results, phosphorylation of Rb was found to be decreased in miR-186–overexpressing cells but increased in the miR-186–inhibited cells. Furthermore, RNA immunoprecipitation (RIP) analysis following miR-186 transfection showed that mRNAs of cyclin D1, CDK2, and CDK6 could be specifically recruited to the miRNP complex isolated using anti-Ago1 antibody (Fig. 4C). Moreover, we found that transfection of miR-186 mimic oligonucleotides dose-dependently abrogated the expression of luciferase, and such suppressive effects could be reversed by anti-miR-186 oligonucleotides (Fig. 4D). However, mutating 4 nucleotides in the seed sequence of miR-186 mimic oligonucleotides completely abolished their binding to the target 3′-UTRs. Taken together, we concluded that miR-186 was able to directly target the 3′-UTRs of the genes of cell-cycle regulators cyclin D1, CDK2, and CDK6.

To elucidate the functional significance of miRNA-mediated suppression of cyclin D1, CDK2, and CDK6 in the induction of cell-cycle arrest, we reexpressed open reading frames (ORF, without their respective 3′-UTRs) of the 3 genes in miR-186–overexpressing cells and assessed the cell-cycle distribution of these transfectants by flow-cytometry analysis. Unexceptionally, restoration of cyclin D1, CDK2, or CDK6 partially, but significantly, rescued the G1–S transition impaired by miR-186 (Fig. 4E), suggesting that downregulation of cyclin D1, CDK2, and CDK6 were functionally important for the inhibitory effect of miR-186 on NSCLC cell proliferation.

**miR-186 inhibited NSCLC tumor growth in vivo**

The in vitro data that miR-186 negatively modulated NSCLC cell cycle and proliferation prompted us to ask whether the miRNA acted as an endogenous antitumor factor in vivo. A bioluminescent imaging method (26) was used to visualize and quantify the dynamic growth of subcutaneously xenografted NSCLC tumors over a 35-day time-course. In this study, each experimental mouse bearing miR-186–overexpressing and control NSCLC cells, respectively, on right-or left-side dorsal thigh, began to exhibit conspicuous difference in tumor growth between the 2 sides within the first week, and the difference continued to expand through the experimental endpoint, at which tumors of the control group displayed 3.5-fold higher intensity of emanated bioluminescence than the miR-186–overexpressing tumors (Fig. 5A and B). In parallel, this trend
was also confirmed by the sizes and weights of dissected tumors (Fig. 5C and D), strongly suggesting a markedly decelerated proliferation of the tumor cells by miR-186. In consistence, the proportions of proliferative Ki-67-positive cells, as well as staining signals for cyclin D1, CDK2, and CDK6, in miR-186-overexpressing tumors, were substantially lower than those of in control tumors. It was also noteworthy that the resultant tumors overexpressing miR-186 exhibited far more differentiated, less aggressive morphologic appearance, as opposed to the control tumors that displayed poorly differentiated morphology as typical adenocarcinoma (Fig. 5E, left). In addition, in consistence with the results obtained from the cell line models, cyclin D1, CDK2, and CDK6 in miR-186-overexpressing tumors were validated to be lower than those in the vector-control tumors (Fig. 5E, right). These in vivo data strongly supported the notion that miR-186 biologically functioned as a cell-cycle inhibitor and tumor suppressor, and that downregulation of miR-186 contributed to development and progression of NSCLC tumors.

Clinical relevance of miR-186 downregulation in human NSCLC

To examine whether the biologic effects of miR-186 downregulation on NSCLC cell proliferation was clinically relevant, we examined the correlation of miR-186 expression with the protein levels of cyclin D1, CDK2, and CDK6 in clinical specimens of NSCLC paired with noncancerous para-tumor tissues. As shown in Fig. 6A, significantly diminished expression of miR-186 in conjunction with elevated levels of cyclin D1, CDK2, and CDK6 were found in the NSCLC lesions in comparison with those in tumor-adjacent noncancerous tissues. Such an inverse correlation, as Fig. 6B showed, was quantitatively confirmed by our immunostaining assay, which showed that the extent of upregulated expression of cyclin D1, CDK2, and CDK6 (denoted by relative gray value) inversely correlated with the degree of miR-186 downregulation, suggesting that the inhibitory effect of miR-186 on the cyclin D1, CDK2, and CDK6 targets were clinical relevant in NSCLC.

Discussion

The current study has indicated that miR-186 is a tumor-suppressive miRNA in NSCLC, and that multiple cell-cycle promoters, namely, cyclin D1, CDK2, and CDK6, are functional targets of miR-186. Our data present the first demonstration that miR-186 is remarkably downregulated in NSCLC cell lines and surgically excised NSCLC tumors. In this context, we have found that experimental restoration of miR-186 expression in NSCLC cells leads to suppression of cyclin D1, CDK2, and CDK6, cell-cycle arrest at G1–S checkpoint, and disrupted proliferation of the cancer cells, whereas completely silencing miR-186 further upregulates cyclin D1, CDK2, and CDK6 and promotes cell-cycle progression. Furthermore, inverse correlation between miR-186 level and expression of cyclin D1,
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CDK2, or CDK6, as well as tumor progression, is evidenced in our animal experiments and clinical relevance study. Particularly, miR-186 overexpression is significantly associated with improved survival in patients with NSCLC, further suggesting a tumor-suppressive function of the molecule. Interestingly, while little is known about the role of miR-186 in human cancers, miR-186 is among a unique set of 8 upregulated miRNAs in pancreatic cancer (27). Moreover, a study by Myatt and colleagues suggests that miR-186 represses the expression of tumor suppressor gene FOXL1, and thus might contribute to endometrial tumorigenesis, suggesting an oncogenic role of miR-186 in the cancer type (28). On the other hand, however, Lv and colleagues unveiled deletion of miR-186 in 15% of 48 primary medulloblastomas, which implicates a tumor-suppressive function of the miRNA (29). By conducting the current study, we provide compelling biologic as well as clinical evidence that in NSCLC miR-186 expression is markedly downregulated, and that miR-186 acts to suppress the proliferation of NSCLC cells both in vitro and in vivo, suggesting that miR-186 plays a tumor suppressive role in the cancer type. These findings together indicate a dual role of miR-186 as either a tumor-promoting or -suppressive miRNA, underscoring the need to define the specific role of a miRNA in a certain type of cancer. In this context, genetic and microenvironmental cues are believed to be key to determining whether, and how, a miRNA molecule functions to promote or to suppress oncogenesis. Thus, it remains important to thoroughly understand the molecular mechanisms mediating the differential biologic effects and targets of miR-186 in NSCLC and other cancer types.

Our current study has identified 3 central cell-cycle regulators, namely, cyclin D1, CDK2, and CDK6, as bona fide targets of miR-186. These genes sequentially control cell-cycle checkpoints and thus have been implicated as proto-oncogenes and attractive therapeutic targets against cancer. Of note, overexpression of cyclin D1 is one of the most common hallmarks of various cancer types, due to its relatively high amplification frequency (15%–40%) and upregulated expression of its mRNA and protein in a variety of tumors including NSCLC, breast cancer, melanoma, and pancreatic cancer (30). Conditional cyclin D1 overexpression in the mammary gland of transgenic mice is sufficient for developing mammary hyperplasia and breast cancer (31). Ablation of cyclin D1 specifically protects mammary tissues from malignancy (32). Gautschi and colleagues also has suggested that cyclin D1 overexpression represents as a pivotal cause of the malignant transformation in the lung tissues (9). CDK6, which have been found amplified or overexpressed in several malignancies including glioma, sarcoma, lymphoma, and leukemia, is hyperactivated to promote cell proliferation and block differentiation during oncogenesis (33, 34). CDK6 is also found to be elevated in NSCLC and closely relevant to lung cancer development (35). Although rare genetic or epigenetic alterations of CDK2 are reported in human tumors (36), CDK2 has been an attractive therapeutic target for pharmacologic intervention, and small-molecule inhibitor targeting CDK2 impedes NSCLC cell proliferation in vitro and tumor formation in vivo (30, 37). It is very likely, therefore, that cyclin D1, CDK2, and CDK6 might act cooperatively to initiate or promote tumor development and progression, and simultaneous silencing of all 3 genes might represent an effective and efficient strategy of suppressing oncogenesis because a cell that has lost the expression of one of them may still be able to proliferate (38, 39). Hence, identification of miR-186 as an efficient suppressor of cyclin D1, CDK2, and CDK6 provide new insights in our need to develop more efficacious therapeutic approach to improved treatment of NSCLC. On the other hand, however, the mechanism via which miR-186 is downregulated in NSCLC remains uninvestigated. It is of note that the coding sequence of miR-186 is located at chromosome Ip31.1, which also hosts HIF1 gene. Previously, Tsai and colleagues found high frequencies (66.7% and 70.8%) of allelic loss and microsatellite instability (87.5% and 95.2%) of the HIF1 locus at chromosome Ip31.1 in NSCLC (20), suggesting that miR-186 downregulation in NSCLC might be associated with genomic deletion. Consistently, 15% of medulloblastoma cases showed deletion of the miR-186 locus, according to Lv and colleagues’ report (29). Meanwhile, by using a bioinformatic tool (UCSC genome browser) for DNA methylation analysis, we found that the promoter of miR-186 might be a CpG-rich region. Taken together, it would be of great interest to further investigate whether miR-186 downregulation in NSCLC was attributable to genomic deletion and/or promoter methylation.

In summary, on the backdrop that NSCLC remains a highly challenging and deadly malignancy, in addition to searching for novel therapeutic agents, it is equally important to identify effective biomarkers for diagnosis and prognosis of NSCLC. To this end, our present finding that the level of miR-186 expression correlates with the clinicopathologic features and patient survival in NSCLC makes it a reasonable candidate biomarker for determination of the prognosis of the disease. Thus, these findings warrant further investigation on the potential development of miR-186–based prognostic and therapeutic approaches.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: M. Li

Methodology: J. Wu, H. Zhang, Y. Yang, X. Zhu

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. J. Cai, L. Fang, Y. Huang, R. Li

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Cai, J. Wu, H. Zhang, L. Fang, Y. Huang, Y. Yang, X. Zhu, R. Li

Writing, review, and/or revision of the manuscript: J. Cai, M. Li

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Wu, H. Zhang, Y. Yang, X. Zhu

Study supervision: M. Li

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