MicroRNA-7 Compromises p53 Protein-dependent Apoptosis by Controlling the Expression of the Chromatin Remodeling Factor SMARCD1*

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We previously demonstrated that the epidermal growth factor receptor (EGFR) up-regulated miR-7 to promote tumor growth during lung cancer oncogenesis. Several lines of evidence have suggested that alterations in chromatin remodeling components contribute to cancer initiation and progression. In this study, we identified SMARCD1 (SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily d, member 1) as a novel target gene of miR-7. miR-7 expression reduced SMARCD1 protein expression in lung cancer cell lines. We used luciferase reporters carrying wild type or mutated 3’ UTR of SMARCD1 and found that miR-7 blocked SMARCD1 expression by binding to two seed regions in the 3’ UTR of SMARCD1 and down-regulated SMARCD1 mRNA expression. Additionally, upon chemotherapy drug treatment, miR-7 down-regulated p53-dependent apoptosis-related gene BAX (BCL2-associated X protein) and p21 expression by interfering with the interaction between SMARCD1 and p53, thereby reducing caspase3 cleavage and the downstream apoptosis cascades. We found that although SMARCD1 sensitized lung cancer cells to chemotherapy drug-induced apoptosis, miR-7 enhanced the drug resistance potential of lung cancer cells against chemotherapy drugs. SMARCD1 was down-regulated in patients with non-small cell lung cancer and lung adenocarcinoma cell lines, and SMARCD1 and miR-7 expression levels were negatively correlated in clinical samples. Our investigation into the involvement of the EGFR-regulated microRNA pathway in the SWI/SNF chromatin remodeling complex suggests that EGFR-mediated miR-7 suppresses the coupling of the chromatin remodeling factor SMARCD1 with p53, resulting in increased chemo-resistance of lung cancer cells.

Lung cancer, particularly non-small cell lung cancer (NSCLC), is the leading cause of cancer-related death worldwide. Surgery, radiation, chemotherapy, and targeted therapy are the four primary approaches to treat lung cancer. For patients with advanced stage NSCLC, adjuvant chemotherapy regimens can prolong survival and improve their quality of life (1, 2). Systemic chemotherapy is usually administered through intravenous injection or orally. Although chemotherapy has improved lung cancer therapy, the accompanying drug resistance and cancer recurrence have led to major treatment failures (3, 4). Multiple pathways have been associated with drug resistance, including increased drug efflux, drug inactivation and/or sequestration, and DNA repair and apoptosis defects (5). Because drug resistance and cancer recurrence are the major causes of cancer-related death, their prevention has become a main topic in lung cancer therapy.

MicroRNAs (miRNAs) are small endogenous noncoding RNAs that are ~22 nucleotides in length. MicroRNAs regulate gene expression by binding to complementary sequences in mRNAs primarily localized to the 3’ UTR (untranslated region), thereby post-transcriptionally preventing their protein product synthesis (6). MicroRNAs act as oncogenes (oncomiR) or tumor suppressors in accordance with their target gene functions. Because of their ability to fine-tune protein expression, miRNAs are emerging as key regulators of cancer progression (7). Moreover, accumulating evidence has supported the important roles of miRNAs in increasing anti-apoptosis and drug resistance (8, 9).

The chromatin remodeling complex has long been associated with the transcriptional regulation of nuclear factors. The ATP-dependent SWI/SNF chromatin remodeling complex is a multisubunit complex that plays essential roles in development processes and tumor repression (10). Several components of the SWI/SNF complex have been suggested to possess intrinsic tumor suppressive activity. For example, in vivo conditional inactivation of SNF5 predisposes the individual to aggressive cancer and rapid cancer onset at a median of 11 weeks (11). The ATPase subunit of the SWI/SNF complex (BRG1, or brahma-related gene 1) is frequently mutated or lost in human cell lines.

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3 The abbreviations used are: NSCLC, non-small cell lung cancer; EGFR, epidermal growth factor receptor; qPCR, quantitative PCR; miRNA, microRNA; PAX, paclitaxel; FL, full-length; DM, double mutant.
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and primary tumors. A total of 30% of human non-small cell lung cancer cell lines lack BRG1 expression, and patients with such tumors have a poor prognosis (12).

Epidermal growth factor receptor (EGFR) signaling plays an essential role in epithelial cell proliferation and maintenance. The genetic amplification or mutation of EGFR has been associated with most lung cancers, especially non-small cell lung cancers (13). Although the importance of EGFR signaling in lung cancer progression is well recognized, little is known about the mechanism underlying the involvement of miRNAs in EGFR-mediated cell proliferation and lung tumor progression. We previously identified an evolutionarily conserved regulatory network of EGFR-induced miR-7 expression that targeted Ets2 repressor factor down-regulation to modulate human lung cancer cell growth (14). In this study, we demonstrated that miR-7 targets the chromatin remodeling factor SMARCD1. SMARCD1 (SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily d, member 1) is a member of the SWI/SNF chromosomal remodeling complex and has been shown to associate with several nuclear proteins, such as glucocorticoid receptor and AP1 (15, 16). Recently, SMARCD1 has been shown to interact with p53 and is required for the activation of the p53-associated apoptosis pathway (17). p53 is an important tumor suppressor protein that mediates the stress-induced apoptosis cascade through transcriptional activation of its downstream apoptosis-associated genes (18). Most chemotherapy and cancer target therapies involve the activation of the p53-associated apoptosis pathway (19, 20). Abnormal down-regulation of p53 activity has been associated with poor prognosis and multiple drug resistance (21). Therefore, we examined the functional role of miR-7 in modulating the chromatin remodeling complex and the p53-related drug resistance/anti-apoptotic pathway in human lung cancer. Our results showed that miR-7 inhibited SMARCD1 expression by targeting the 3′UTR of SMARCD1 and reduced the transcriptional activity of the p53-SMARCD1 complex, thereby interfering with the p53-p21-related apoptosis pathway and enhancing lung cancer cells drug resistance.

Experimental Procedures

Cell Culture—A549, H1299, H1975, HCC827, and HEK293T cell lines were obtained from the American Type Culture Collection (ATCC). All lung cancer cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 50 units/ml penicillin, and 50 μg/ml streptomycin. HEK293T cells were cultured in DMEM supplemented with 10% FBS, 50 units/ml penicillin, and 50 μg/ml streptomycin.

Plasmid Constructs—Lentiviral miR-7 overexpression plasmids were constructed as described previously (14). In brief, miR-7 was cloned from 500-bp flanking sequences of CL1–5 human genomic DNA into the HR’-puro lentivector vector. HR’-puro-SMARCD1(FL) (containing full-length 3′UTR) plasmid was constructed by inserting PCR-amplified SMARCD1 sequence into HR’-puro vector. Primers used for PCR amplification of SMARCD1 were as follows: forward, 5′-GGATTCCTGATTGAGTCGACGGC-3′, and reverse, 5′-GTCGACTCTTCTTTGCTTATGGTATTGAAAGGAAGA-3′. The 3′UTR of SMARCD1 corresponding to 15–1713 was PCR-amplified and subcloned into the 3′ region of luciferase gene in pGL3-control vector (Promega) using two primers as follows: forward-15′, 5′-GGATTCCTGATTGAGTCGACGGC-3′, and reverse-1713′, 5′-GTCGACTCTTCTTTGCTTATGGTATTGAAAGGAAGA-3′. This plasmid containing wild type 3′UTR of SMARCD1 was named as pGL3-SCD1–3′UTR-luc. The pGL3-SCD1–3′UTR-luc plasmid was then used as the template to generate three SMARCD1–3′UTR mutant plasmids as shown in Fig. 2C, top panel (termed as 3′UTR-M1, 3′UTR-M2, and 3′UTR-DM, respectively) using the QuickChange® site-directed mutagenesis kit (Stratagene) according to the manufacturer’s standard protocol. The primers used to generate the point mutations were designed with the QuickChange Primer Design Program. The primer sequences used to generate mutant 1 (3′UTR-M1) were sense 5′-CTGGGCACTACCTGTTGTTTCTGTCCCTTGTCTGC-3′ and antisense 5′-GCAAGCAGCAGACAAACACAGGATGGCCCAAG-3′. The primer sequences used to generate mutant 2 (3′UTR-M2) were sense 5′-TTTCCAGGAGAGCCCATCTTCTTCTGTGTCAGGTTGTATCAC-3′ and antisense 5′-GTGATACACCAGGAAAGAATTGGAGCTGCTCCTCGGAAA-3′. Double seed region mutant clone (3′UTR-DM) was generated in the sequential mutation of 3′UTR-M1 and 3′UTR-M2. pCDNA3-p53 was constructed by insertion of PCR-amplified p53 fragment into pCDNA3.0 vector (Addgene). Primers used for PCR amplification of p53 were as follows: forward, 5′-GGGTCACTGCCATGGAGGA-3′; reverse, 5′-CAGGTTGTATCAC-3′.

Lentivirus Package and Infection—Lentivirus preparation and infection were performed as described (22). In brief, 2 × 10^6 HEK293T cells were seeded into a 100-mm culture plate for 24 h, and the medium was replaced with fresh medium 2 h before transfection. Using a standard calcium phosphate transfection protocol, 9 μg of ΔΔ9.3 plasmid and 2.5 μg of vesicular stomatitis virus G protein plasmid, together with 10 μg of the pLKO.1-based lentiviral vector or HR’-puro-based lentiviral vector, were co-transfected into HEK293T cells. The medium was replaced with fresh medium 16 h later, and virus-containing supernatants were collected 40 h after transfection. Lenti-viral infection was carried out by adding virus solution to the cells in the presence of Polybrene (8 ng/ml). 24 h later, cells were refreshed with medium containing puromycin (2 μg/ml). Cells were selected, and cells that survived over 48 h were cultured for future experiments.

RNA Interference—All pLKO-based shRNA clones for SMARCD1 and p53 were purchased from the National RNAi Core Facility at Academia Sinica (Taipei, Taiwan). The sequences of human SMARCD1 siRNA are si-SCD1-1, 5′-GATTTTTGAGTCTCACAAGTAT-3′, and si-SCD1-2, 5′-CAGCTGCAACCAATTTTTGATT-3′. The sequences of human p53 siRNA are si-p53-1, 5′-CACCATTCACCATCACACATCAT-3′, and si-p53-2, 5′-CGCGCGCAGAGGAAGAAGAAT-3′. Virus was generated, infected, and selected as aforementioned.

WST-1 Cell Viability Assay—A total of 5 × 10^3 A549-derived stable cells were seeded into a 96-well culture plate. After 24 h, the media were replaced with complete media containing various concentrations of cisplatin (Sigma) for an additional 24 h.
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The number of surviving cells was determined by a standard colorimetric WST-1 assay (Roche Applied Science).

Transfection and Luciferase Assays—A total of 1 × 10⁵ A549 cells were seeded into a 24-well culture plate. After 24 h, the media were replaced with fresh media, and the cells were transfected with 500 ng of plasmids containing reporter plasmids (100 ng) and test plasmids (200 ng) according to experimental design. PRL-SV40 (5 ng) was used as an internal transfection efficiency control. All transfections were performed with Lipofectamine 2000 (Life Technologies, Inc.) following the standard transfection protocol. Cell lysates were collected 48 h after transfection and subjected to the Dual-Luciferase® reporter assay system (Promega) following the standard protocol.

Annexin-V FITC Apoptosis Assay—A total of 3 × 10⁵ cells were seeded into a 60-mm culture plate for 24 h. The media were then replaced with media containing desired concentrations of cisplatin or other chemotherapy drugs. After 24 h, the supernatant was collected, and attached cells were detached and analyzed for apoptosis index. Annexin-V FITC and propidium iodide staining were performed using the annexin V: FITC apoptosis detection kit I (BD Biosciences) following the standard protocol. The apoptosis index was determined with a BD FACSCanto™ II Cell Analyzer (BD Biosciences).

mRNA Extraction and Real Time Quantitative PCR—Total RNA of cultured cell lines and fresh-frozen tissues from normal tumor-paired patient samples were collected and lysed with TRIzol reagent (Invitrogen). Total RNA was extracted following the manufacturer’s instructions. 1 μg of total RNA was used for reverse transcription using NCode miRNA first-strand cDNA synthesis and qPCR kits (Life Technologies, Inc.). The Universal reverse qPCR primer for microRNA detection was included in the kit. MicroRNA expression of U6 snRNA and miR-7 and mRNA expression of p21, BAX, Puma, Noxa, SMARCD1, and GAPDH was detected by TOOLS 2xSYBR qPCR MIX (BIOTOOLS, Taiwan). Forward primers used for microRNA detection are as follows: U6 snRNA forward primer, 5′-ACGCAATTCGTGAAGCCTTCCAT-3′; miR-7 forward primer, 5′-CGTGGGAGAGGATGATGTTTGT-3′. Primers used for mRNA detection are as follows: GAPDH forward, 5′-CGGACACTTGTGTGCAGCTA-3′; and GAPDH reverse, 5′-AGGCTTCACTGGGCAAACGTG-3′; SMARCD1 forward, 5′-TTGCGGTAAGAAGCGG-3′; and SMARCD1 reverse, 5′-TTGTTATTGTGGCATCATATTTGGG-3′; p21 forward, 5′-TGTTCCGTCAGACCAACA-3′; and p21 reverse, 3′-CGAAGTTCTCCTGCATC-3′; BAX forward, 5′-GGGTTGGTGAGACTC-3′, and BAX reverse, 5′-AGACACGGTAAGGGAAACGCATTA-3′; Noxa forward, 5′-GAGATGCCTGGGAAAGGAAGG-3′; and Noxa reverse, 5′-TCTGTGCCGAAGTTACATTTTTG-3′; Puma forward, 5′-AGACACTTCAACGCACACTG-3′, and Puma reverse, 5′-TGGGTTAAGGCGAGGATCTG-3′.

Patient Samples—A total of 30 NSCLC patient samples were obtained according to the Taipei Medical University Institutional Review Board-approved guidelines. Tumor tissues and adjacent normal tissues were freshly frozen in liquid nitrogen and stored at −80 °C until use. 20 patients were diagnosed with lung adenocarcinoma and 10 patients were diagnosed with squamous cell lung carcinoma according to the classification guidelines from the World Health Organization. The EGFR mutation status was determined by a direct sequencing method (exons 18–21 in EGFR) (14). 40 samples from patients with lung adenocarcinoma were obtained under the auspices of the Institutional Review Board of the National Health Research Institute. Their tumor types were determined according to the classification guideline from the World Health Organization. Tumor tissues were freshly frozen in liquid nitrogen and stored in −80 °C. The EGFR status was not determined in these samples. The p53 status was not determined in both origins of patient samples.

Antibodies—For immunoblot analysis, protein samples were resolved by SDS-PAGE and transferred to nitrocellulose membrane (Millipore). Anti-cleaved caspase-3 (catalog no. 9665, Cell Signaling), anti-p53 (catalog no. 9282, Cell Signaling), anti-SMARCD1 (sc-135843, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-BAX (sc-493; Santa Cruz Biotechnology, Inc.), and anti-α-tubulin (GTX112141, GeneTex) antibodies were purchased commercially and applied to perform Western blot analysis according to standard protocols.

miR-7 Sponge Generation—Sense and antisense miR-7 sponge oligonucleotides (together with sense and antisense linker oligonucleotides) were annealed, ligated, gel-purified, and subcloned into the HR′-puro plasmid, as described previously (23, 24). Colonies were screened by PCR after transformation and subjected to DNA sequencing. The oligonucleotide sequences were as follows: miR-7 sponge F′, 5′-ACAACAAAAATCACTAGTCTTCCAGG-3′, and miR-7 sponge R′, 5′-TGGGAGACTGATGTTTGTGTCG-3′; Linker F′, 5′-CGTGAGTCCAGATCTCGG-3′, and Linker R′, 5′-GAGATCGTCCAGTCGACCC-3′.

MicroRNA Targeting Prediction—The miR-7 targeting SMARCD1 was analyzed by the on-line starBase version 2.0 database. The starBase version 2.0 is an on-line web-based prediction program for systematically identifying the RNA-RNA and protein-RNA interaction networks, including several prediction programs. Default setting was applied to check whether SMARCD1 is targeted by miR-7. The miR-7 seed region in 3′UTR of SMARCD1 was predicted by the aid of microRNA target prediction website TargetScanHuman. SMARCD1 and has-miR-7–5p were input as gene symbol and microRNA name, and the results were shown after executing the program.

Statistical Analysis—The data are presented as the mean ± S.D. qRT-PCR analysis of SMARCD1 was performed with 30 pairs of primary NSCLC samples and their matching adjacent non-tumorous lung tissues. Statistical significance was calculated using the Wilcoxon test. The Pearson Product-Moment Correlation Coefficient was used to compare miR-7 and SMARCD1 expression in 70 NSCLC samples.

Results

Ectopic Expression of miR-7 Protects A549 Lung Cancer Cells from Cisplatin-induced Cell Death—We previously showed that ectopic expression of miR-7 inhibited Ets2 repressor factor expression and thus promoted lung cancer oncogenesis, suggesting that miR-7 acts as an oncomiR (14). We were interested in whether miR-7 participated in regulating chemotherapy-induced cell death, as drug resistance is an important issue in lung
cancer therapy. Apoptosis is an important pathway involved in chemotherapy-induced cell death. We examined whether miR-7 participated in lung cancer cell apoptosis. We used the A549 lung adenocarcinoma cell line, which expresses endogenous wild type p53 and has a functional p53-dependent apoptotic pathway, to examine miR-7 function in the regulation of apoptosis. We infected A549 cells with a lentivirus bearing an miR-7-overexpressing sequence and then treated them with 50 μM cisplatin for 24 h to determine the apoptotic index. Annexin V-FITC staining showed that A549/miR-7 cells had a lower apoptotic index (7.36%) than that of A549/ctrl cells (14.94%) upon cisplatin exposure (Fig. 1A, p < 0.05). TUNEL assays also showed similar results (Fig. 1B). These data indicated that miR-7 expression enhances A549 cell anti-apoptosis ability. To test whether miR-7 expression influenced the drug response of A549/miR-7 and A549/ctrl cells, we treated both cell lines with various doses of cisplatin. The results showed that compared with A549/ctrl cells, A549/miR-7 cells were more resistant to cisplatin treatment (Fig. 1C). Moreover, miR-7 expression reduced cisplatin-induced caspase3 activation (Fig. 1D). These results may partially explain how miR-7 inhibits drug-induced apoptosis. Thus, miR-7 may have an anti-apoptosis function in lung cancer cells.

miR-7 Targets the 3′ UTR of SMARCD1 and Suppresses Its Expression—p53 is a critical mediator of the apoptosis signaling pathway and acts as a tumor suppressor in several cancer cells (18, 25). Therefore, we focused on p53-associated apoptosis regulating factors that could be targeted by miR-7. In our previous study, we identified several putative miR-7 target proteins (14). We further employed three microRNA target prediction

FIGURE 1. miR-7 overexpression protects A549 cells from cisplatin-induced apoptosis. A, A549 cells were infected with a lentivirus carrying the HR_puro control vector (A549/ctrl) or HR_puro-miR-7 plasmid (A549/miR-7). A549/ctrl and A549/miR-7 cells were treated with 50 μM cisplatin for 24 h and then subjected to the annexin V-FITC assay to determine apoptotic cell ratios. B, A549/ctrl and A549/miR-7 cells were treated with 50 μM cisplatin for 24 h and then subjected to TUNEL assays. C, A549/ctrl and A549/miR-7 cells were treated with various doses of cisplatin as indicated. The cells were subjected to WST-1 proliferation assays after 48 h. D, A549/ctrl and A549/miR-7 cells were treated with 50 μM cisplatin for 24 h as indicated. The expression level of cleaved caspase3 and p53 was determined by Western blotting. α-Tubulin was used as an internal loading control. *, p < 0.05; **, p < 0.01. All results are presented as the average of triplicate experiments. Ctrl, control.
programs (PICTAR, Target Scan, and PITA) on the on-line starBase version 2.0 database to examine these putative miR-7 target proteins (26, 27). All three programs identified the chromatin remodeling complex factor SMARCD1 as an miR-7 target (Fig. 2, top panel). SMARCD1 is a component of the chromatin remodeling complex. A recent report showed that SMARCD1 interacted with p53 and was required for the recruitment of the chromatin remodeling complex to form the p53-dependent transcription complex. Down-regulation of SMARCD1 attenuated p53-mediated transcription (17). After infecting with lentiviruses carrying HR'-puro-miR-7 and HR'-puro control vector into three lung adenocarcinoma cell lines, A549, H1975, and HCC827 cells, we performed Western blot analysis to examine SMARCD1 expression. The result demonstrated that miR-7 overexpression reduced SMARCD1 expression in all three cell lines (Fig. 2, lower panel).

We next examined whether miR-7 targeted the 3'UTR of SMARCD1 mRNA. Two putative miR-7-binding sequences in the 3'UTR of SMARCD1 are illustrated (top panel). Two single-site mutants (3'UTR-M1 and 3'UTR-M2) and one double-site mutant (3'UTR-DM) in the SMARCD1 3'UTR were generated as indicated by the arrows using pGL3-SCD1–3'UTR-luc as the template. Luciferase reporter analyses were performed in A549 cells transiently transfected with the luciferase reporter plasmid containing the wild type SMARCD1 3'UTR (3'UTR-WT) or one of the three mutants (3'UTR-M1, 3'UTR-M2, and 3'UTR-DM), together with either the HR'-puro (Ctrl) or HR'-puro-miR-7 (miR-7) plasmids. The Renilla luciferase plasmid pRL-SV40 was used as the transfection efficiency control. All results are presented as the average of triplicate experiments.

D, miR-7 and SMARCD1 mRNA expression was determined using SYBR Green qPCR in control or miR-7-overexpressing A549, H1975, and HCC827 cells. For miR-7 quantification, U6 snRNA was used as an internal control; for SMARCD1 quantification, GAPDH was used as an internal control. All results are presented as the average of triplicate experiments.

FIGURE 2. miR-7 inhibits SMARCD1 expression by binding to two complementary sites in the 3’UTR of SMARCD1 mRNA. A, top panel, miR-7 targets were predicted by the TargetScan, picTar, and PITA programs on the on-line starBase version 2.0 database using the default parameters setting. Lower panel, immunoblots were used to measure SMARCD1 expression in A549, H1975, and HCC827 lung cancer cells 5 days after infection with a lentivirus bearing HR'-puro (Ctrl) or HR'-puro-miR-7 (miR-7). Ectopic miR-7 overexpression reduced SMARCD1 expression in A549, H1975, and HCC827 cells. The SMARCD1 expression ratio between control cells and miR-7-overexpressing cells was normalized to α-tubulin, which served as the internal loading control. B, pGL3-SCD1–3’UTR-luc reporter plasmid was used to monitor miR-7 targeting of SMARCD1. The luciferase reporter activity of pGL3-SCD1–3’UTR-luc was decreased upon miR-7 overexpression, but no change was observed in cells expressing the pGL3-control reporter plasmid (pGL3-ctrl). The Renilla luciferase plasmid pRL-SV40 was used as the transfection efficiency control. The results are presented as the average of triplicate experiments. **, p < 0.01. C, miR-7 targets the 3’UTR of SMARCD1. Two putative miR-7-binding sequences in the 3’UTR of SMARCD1 are illustrated (top panel). Two single-site mutants (3’UTR-M1 and 3’UTR-M2) and one double-site mutant (3’UTR-DM) in the SMARCD1 3’UTR were generated as indicated by the arrows using pGL3-SCD1–3’UTR-luc as the template. Luciferase reporter analyses were performed in A549 cells transiently transfected with the luciferase reporter plasmid containing the wild type SMARCD1 3’UTR (3’UTR-WT) or one of the three mutants (3’UTR-M1, 3’UTR-M2, and 3’UTR-DM), together with either the HR'-puro (Ctrl) or HR'-puro-miR-7 (miR-7) plasmids. The Renilla luciferase plasmid pRL-SV40 was used as the transfection efficiency control. All results are presented as the average of triplicate experiments. **, p < 0.01. **, p < 0.001.
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3'UTR-luc). We co-transfected A549 cells with the pGL3-SCD1–3'UTR-luc reporter and HR'-puro-miR-7, and the luciferase reporter activity was measured 48 h after transfection. The results showed that miR-7 expression down-regulated the luciferase activity of the reporter plasmid containing the 3'UTR of SMARCD1 but did not affect the pGL3-control vector (Fig. 2B). These results suggested that miR-7 down-regulated SMARCD1 by targeting its 3'UTR. With the aid of the microRNA target prediction website TargetScanHuman, we identified two possible miR-7-binding sites in the 3'UTR of SMARCD1 (Fig. 2C, top panel). We constructed three mutated clones within the 3'UTR of SMARCD1 by generating point mutations in each putative miR-7 seed region and both seed regions using pGL3-SCD1–3'UTR-luc plasmid as a template. Three mutation constructs were named as 3'UTR-M1, 3'UTR-M2, and 3'UTR-DM, respectively (Fig. 2C, top panel). We found that miR-7 expression attenuated the luciferase activity of pGL3-SCD1–3'UTR-luc. Single mutant clones (3'UTR-M1 and 3'UTR-M2) partially rescued reporter activity, whereas the double mutant clone (3'UTR-DM) was not significantly affected by miR-7 expression. These results suggested that miR-7 down-regulated SMARCD1 expression by binding to the two seed regions in its 3'UTR (Fig. 2C, lower panel). We further examined whether miR-7 affected SMARCD1 mRNA expression. The results showed that upon miR-7 overexpression, SMARCD1 mRNA decreased in A549, H1975, and HCC827 cells. Thus, miR-7 inhibited SMARCD1 expression through targeting the 3'UTR of SMARCD1.

SMARCD1 Promotes Drug-induced Apoptosis and miR-7 Attenuates the Effect of SMARCD1 in Lung Cancer Cells—We examined the possible role of SMARCD1 in the drug response in lung cancer cells. We infected A549 cells with a lentivirus carrying control vector (HR), miR-7, SMARCD1(FL), and SMARCD1(FL) + miR-7 in H1975 cells. H1975 cells were infected with lentivirus carrying control vector (HR), miR-7, SMARCD1(FL), and SMARCD1(FL) + miR-7, respectively. We exposed these four cell lines to 50 μM cisplatin for 24 h to determine their apoptotic index. Upon cisplatin treatment, SMARCD1 overexpression up-regulated caspase3 activation, miR-7 co-expression attenuated SMARCD1-induced apoptosis (Fig. 3D). Annexin V-FITC staining showed similar results as in A549 cells. SMARCD1 overexpression increased the cisplatin-induced apoptosis ratio, and further addition of miR-7 attenuated SMARCD1-enhanced cell death (Fig. 3E, p < 0.05).

To further confirm the ability of SMARCD1 to increase apoptosis, we used two shRNA clones to knock down endogenous SMARCD1 expression in A549 and H1975 cells. Upon cisplatin treatment, the two SMARCD1 shRNA clones down-regulated caspase3 activation and the total apoptosis ratio (Fig. 3, F and G). Taken together, our results demonstrated that SMARCD1 enhances cisplatin-induced cell death and miR-7 attenuates SMARCD1 function.

miR-7 Attenuates p53/SMARCD1-mediated Apoptosis—The pro-apoptotic protein BAX is a direct p53 transcriptional target and is involved in the p53-mediated apoptosis signaling pathway (25). We investigated whether miR-7 and SMARCD1 affected BAX expression. We overexpressed miR-7 and SMARCD1 in A549 and H1975 cells to examine whether miR-7 and SMARCD1 regulated p53-dependent apoptosis in lung cancer cells. The results showed that BAX expression decreased upon SMARCD1 reduction in miR-7-overexpressing A549 and H1975 cells (Fig. 4A, 1st and 3rd panels), but BAX expression did not significantly change in p53-null H1299 cells (Fig. 4B, 1st panel). SMARCD1 overexpression increased BAX expression in A549 and H1975 cells (Fig. 4A, 2nd and 4th panels) but did not affect BAX expression in H1299 cells (Fig. 4B, 2nd panel). We further examined miR-7 and SMARCD1 function in H1299 cells by adding back functional wild type p53. The results showed that after adding back p53 into H1299, miR-7 expression down-regulated BAX expression (Fig. 4B, 3rd panel). These data suggested that miR-7 down-regulates BAX expression in a p53-dependent manner.

We next examined the effect of miR-7 on the activity of the p53-dependent transcriptional complex. A commercially available pG313-luc plasmid (Addgene) containing 13 repeats of the p53-binding sequence in the pGL3 plasmid promoter region is an artificial reporter used to monitor endogenous p53 transcriptional activity. We transfected the pG313-luc plasmid into A549 cells together with plasmids encoding p53, SMARCD1,
and miR-7. We measured the luciferase reporter activity 48 h after transfection. The reporter assay results showed that p53 overexpression enhanced pG13-luc reporter activity as expected, whereas p53 and SMARCD1 (FL) co-expression further increased pG13-luc reporter activity; moreover, miR-7 co-expression decreased pG13-luc reporter activity (Fig. 4C).

We measured the mRNA levels of four p53-associated pro-apoptotic genes (p21, BAX, Puma, and Noxa) to examine whether miR-7 expression affected their expression. The results showed that miR-7 overexpression down-regulated the mRNA levels of cisplatin-induced apoptosis-related genes (Fig. 4D). These results indicated that miR-7 inhibited SMARCD1 expression and thus down-regulated p53-associated apoptosis gene expression upon cisplatin treatment.

To further elucidate the effect of p53 on miR-7/SMARCD1 in cisplatin-induced apoptosis, we knocked down endogenous p53 using shRNA in A549 and H1975 cells. The results showed that p53 knockdown in both cell lines abolished the inhibitory
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Effect of miR-7 on cisplatin-induced caspase3 activation and apoptosis (Fig. 4, E and F). The results demonstrated that miR-7 inhibits cisplatin-induced apoptosis in a p53-dependent manner.

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Drug in patients with advanced NSCLC due to their apoptosis-inducing ability (28). We exposed A549 and H1975 cells carrying control vector, miR-7, and SMARCD1(FL) to 50 nm PAX treatment for 24 h. We then examined their BAX expression and apoptotic indices. PAX treatment stabilized p53 and up-regulated BAX expression. miR-7 overexpression down-regulated BAX expression, and SMARCD1 overexpression increased BAX expression (Fig. 5A).
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V-FITC staining in A549 and H1975 cells to examine their apoptotic indices. For A549 cells exposed to paclitaxel, we observed an apparent morphological change, and in the protein level we observed the effect of miR-7 and SMARCD1 on apoptosis regulation, but we were unable to observe clearly the apoptotic effect in annexin V-FITC staining assay. It appeared as similar results in the following experiments when using annexin V-FITC staining to examine the apoptotic index of A549 cells exposed to paclitaxel treatment. But in H1975 cells, the results were consistent with Western blotting, i.e. miR-7 expression down-regulated the PAX-induced apoptotic ratio, and SMARCD1 expression increased the apoptotic ratio (Fig. 5B).

We also knocked down endogenous SMARCD1 expression using two shRNA clones against SMARCD1 to confirm SMARCD1 function in PAX-induced apoptosis. The results showed that SMARCD1 knockdown decreased BAX expression and the apoptosis ratio (Fig. 5C and D). We also knocked down p53 in miR-7-overexpressing cells to examine its importance in PAX-induced apoptosis. The results showed that p53 silencing in miR-7-overexpressing cells restored BAX expression and the apoptosis ratio, which was inhibited by miR-7 upon PAX treatment (Fig. 5E and F). These results verified that miR-7 can rescue PAX-induced apoptosis, and SMARCD1 exacerbates PAX-induced apoptosis in a p53-dependent manner.

Anti-miR-7 Sponge Enhances Cisplatin-induced Cell Death—A microRNA sponge is thought to be a competitive inhibitor of endogenous small RNAs in mammalian cells (23). To investigate the effect of miR-7 down-regulation in lung cancer cells, we generated a lentivirus-based system expressing an artificial
miR-7 sponge that contained six repeats of the miR-7-binding sequence to antagonize endogenous miR-7 function. When we infected A549 cells with the lentivirus carrying the miR-7 sponge (A549/miR-7-sponge cells), endogenous miR-7 expression decreased as expected (Fig. 6A). When the cells were exposed to cisplatin treatment (50 μM for 24 h), the miR-7 sponges increased SMARCD1 expression and caspase3 cleavage (Fig. 6A). A549/miR7-sponge cells were tested for their response to cisplatin treatment using the annexin V-FITC assay. The results showed that miR-7-sponge overexpression sensitized the A549 cells to cisplatin-induced early cell death (increase from 6.5 to 15.3%) (Fig. 6B, p < 0.05).

SMARCD1 Expression Is Down-regulated and Negatively Correlated with miR-7 Expression in NSCLC Patients—We examined the correlation between miR-7 and SMARCD1 expression in the following seven lung adenocarcinoma cell lines: A549, H1975, HCC827, CL1–0, CL1–5, H3255, and H928 cells and one normal cell line, Beas2B cells. The results showed that miR-7 expression was higher in the seven lung cancer cells compared with Beas2B cells, and SMARCD1 expression was down-regulated in the seven lung cancer cell lines compared with Beas2B cells (Fig. 6C). We further examined SMARCD1 expression in a cohort study with 30 NSCLC patients to investigate its clinical relevance in lung cancer samples using the
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Wilcoxon signed ranks test. SMARCD1 mRNA was decreased in tumor tissues compared with adjacent normal tissues (Fig. 6D, \( p < 0.05 \)). This result was consistent with our hypothesis that SMARCD1 functions as a tumor suppressor. We further examined the correlation between miR-7 and SMARCD1 expression in 70 NSCLC clinical samples using Pearson’s correlation analysis. The results showed a negative correlation between SMARCD1 and miR-7 (Fig. 6E, \( r = -0.245, p < 0.05 \)), thereby confirming that miR-7 and SMARCD1 were clinically negatively correlated.

Discussion

In this study, we demonstrated that the miR-7 overexpression in A549 cells down-regulated the expression of the apoptosis-related genes BAX and p21 by interfering with the p53/SMARCD1-dependent transcriptional complex, thereby reducing the chemotherapy drug-induced apoptosis effect and enhancing lung cancer cell drug resistance (Fig. 6F). We also observed a similar anti-apoptosis effect for miR-7 in chemotherapy drug-treated H1975 cells, a lung adenocarcinoma cell line expressing a mutant EGFR (Figs. 3–5). Our results suggest that miR-7 can exert its anti-apoptosis effect in lung cancer cells expressing either wild type or mutant EGFR.

Additionally, we examined whether miR-7 targeted other chromatin remodeling factors in addition to SMARCD1 using the on-line starBase version 2.0 database, including PICTAR, TargetScan, and PITA. We did not observe any other chromatin remodeling factor targeted by miR-7. This finding further emphasizes the significance of SMARCD1 in miR-7-mediated lung cancer progression.

We previously demonstrated that miR-7 was overexpressed in primary lung cancers and that EGFR and miR-7 expression levels were significantly correlated in EGFR-mutated lung adenocarcinomas (14). In this study, we further confirmed the oncogenic potential of miR-7 through its association with the chromatin remodeling factors SMARCD1 and p53, thereby influencing anticancer drug resistance. miR-7 was recently reported to have dual functions in both cell cycle arrest and anti-apoptosis in CHO cells (29). miR-7 indirectly down-regulated p53 expression and activated p-Akt in CHO cells to protect cells from apoptosis, indicating that miR-7 plays an elegant role in fine-tuning cell cycle regulation and apoptosis. In this study, we did not find that miR-7 overexpression decreased p53 expression, which may be due to the fundamental genetic differences between CHO cells (a normal cell type) and lung cancer cells. Aside from p53 degradation, we observed different effects on the p53-dependent transcriptional regulation of cell apoptosis. Meza-Sosa et al. (30) reported that the transcription factor KLF4 was a direct miR-7 target and targeting of KLF4 by miR-7 promoted cellular transformation and tumor growth in A549 cells. These data are consistent with our finding that miR-7 acts as an oncomiR in lung cancer cells.

A new circular RNA CDR1 was recently reported to function as a native sponge capable of binding miR-7 in human tissues in a manner similar to miR-7 knockdown (31). Another natural circular RNA ciRS-7 (circular RNA sponge for miR-7) potently suppressed miR-7 expression, resulting in increased protein levels of miR-7 targets (32). In our study, the use of an miR-7 sponge efficiently down-regulated endogenous miR-7 expression, increased SMARCD1 expression, and sensitized A549 cells to cisplatin treatment. This finding indicates that artificial miR-7 sponges may be able to serve as a clinical drug for use in malignant lung cancer cases.

Platinum-based and paclitaxel combination chemotherapy are the primary therapeutic methods for lung cancer patients. Chemotherapy drugs such as cisplatin induce cell growth arrest or cell death in cancer cells, eventually leading to apoptosis activation and other cell death mechanisms (33). However, some patients initially respond well and then gradually develop drug resistance, leading to eventual tumor relapse. Drug resistance in patients with lung cancer is the major cause of a poor 5-year survival rate (less than 15% for NSCLC and 5% for SCLC) (34). The identification of factors involved in drug resistance will provide more targets for cancer therapy. Previous studies have shown that cisplatin treatment resulted in the transcriptional activation of several apoptotic DNA repair and cell cycle regulator genes in an NSCLC cell line (35). Interestingly, we identified a novel pathway involved in cisplatin treatment in NSCLC, especially in chromatin remodeling and assembly. Alterations in the SWI/SNF chromatin remodeling factor have been reported in many tumor types (36). For example, loss of heterozygosity of the region surrounding BRG has been frequently identified in human adenocarcinomas (37). Although repressing the SWI/SNF complex function may provide an excellent tool to alter the gene expression profile of tumor cells and promote tumorigenesis, the underlying mechanisms have not been well defined. In tumor cells, the SWI/SNF chromatin remodeling complex is involved in tumor progression through its interaction with p53. SMARCD1, a core subunit of the SWI/SNF complex, has been shown to function as a mediator between the SWI/SNF complex and p53 (17). In our study, we demonstrated that SMARCD1 enhanced drug-induced apoptosis in the p53-positive lung cancer A549 and H1975 cell lines, and miR-7 expression decreased the death-enhancing ability of SMARCD1. However, in the p53-null cell line H1299, miR-7 can still down-regulate SMARCD1 expression but was unable to reduce BAX expression (Fig. 4B). When wild type p53 was introduced back into H1299 cells, BAX expression was down-regulated by miR-7 expression upon cisplatin exposure. These findings provide evidence that SMARCD1 has to interact with p53 to promote BAX-dependent apoptosis. Notably, miR-7 blocked SMARCD1 translation by targeting the seed region in its 3’UTR, as point mutations in the seed region abrogated miR-7 inhibition of SMARCD1.

Several microRNAs have shown functional correlations with chromatin remodeling complex components. For example, Cai et al. (38) demonstrated that miR-211 overexpression promoted colorectal cancer cell growth by targeting chromodomain helicase DNA-binding protein 5 (CHD5), which was a newly identified chromatin-associated tumor suppressor. Our data showed that miR-7 expression had anti-apoptotic and drug resistance effects by repressing the chromatin remodeling complex/p53 transcriptional activity. Our data provide the first evidence that one microRNA participates in chromatin remodeling complex-mediated transcriptional control in lung cancer cells and therefore the regulation of lung cancer progression.

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In conclusion, our findings demonstrated that miR-7 modulated EGFR-mediated oncogenesis by promoting lung cancer cell growth or reducing the apoptosis index. Thus, miR-7 may serve as an important modulator of EGFR-mediated drug resistance and oncogenesis and provide a novel prognostic biomarker and therapeutic target for lung cancer.

Author Contributions—C.-F. H. performed most of the experiments, collected data, analyzed the results, and wrote the manuscript. S.-Y. L. performed all SILAC experiments and analyzed the results. Y.-T. C. provided the conception, designed experiments, analyzed the results, and revised the manuscript. C.-W. W. integrated all research resources, provided the research directions, and revised and approved the final version of this manuscript. All authors reviewed the results and approved the final version of the manuscript.

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