MicroRNA-101 Exerts Tumor-Suppressive Functions in Non-small Cell Lung Cancer through Directly Targeting Enhancer of Zeste Homolog 2

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Introduction: Overexpression of the enhancer of zeste homolog 2 (EZH2) protein has been found in broad range of cancer types, including non-small cell lung cancer (NSCLC). Nevertheless, the mechanisms by which EZH2 becomes overexpressed in NSCLC remain unclear. MicroRNAs (miRNAs) can regulate target gene expression through translational control. In this study, we investigate whether miRNA (miR-101) regulates EZH2 expression in NSCLC.

Methods: We evaluated the expression of miR-101 and EZH2 in 20 matched NSCLC and adjacent nontumor lung tissues by reverse-transcriptase polymerase chain reaction and immunohistochemistry, respectively. Luciferase reporter assay was used to determine whether miR-101 directly targets EZH2. To assess the effect of miR-101 on NSCLC biological behavior, cell proliferation, invasion, and response to chemotherapy were analyzed using NSCLC cells transfected with miR-101 mimics or transfected with specific small interfering RNA to deplete EZH2 (small interfering RNA-EZH2).

Results: Reduced expression of miR-101 was associated with overexpression of EZH2 in NSCLC tumor tissues. Transfection of miR-101 mimics significantly suppressed the activity of the luciferase reporter containing wild type but not mutant EZH2 3′-UTR and decreased EZH2 expression in NSCLC cell lines. Furthermore, enforced expression of miR-101 or knockdown of EZH2 led to reduced NSCLC cell proliferation and invasion and sensitized cancer cells to paclitaxel-mediated apoptosis through inducing expression of the proapoptotic protein Bim.

Conclusions: miR-101 inhibits cell proliferation and invasion and enhances paclitaxel-induced apoptosis in NSCLC cells, at least in part, by directly repressing EZH2 expression. Therapeutic strategies to rescue miR-101 expression or silence EZH2 may be beneficial to patients with NSCLC in the future.

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Enhancer of zeste homolog 2 (EZH2) is a human homolog of the drosophila polycomb group genes, which are critical for transcriptional regulation by chromatin remodeling, nucleosome modification, and interaction with other transcription factors.[1] EZH2 is believed to function as a histone methyltransferase for lysine 27 of histone H3 (H3K27) by forming a multimeric protein complex, called polycomb repressive complex 2 including embryonic ectodern development and suppressor of zeste 12 homolog.[2] There is increasing evidence that EZH2 possesses oncogenic properties as overexpression of EZH2 enhances proliferation and invasion of cancer cells and promotes neoplastic transformation.[3–5] Oppositely, depletion of EZH2 in cancer cells leads to reduced proliferation, increased apoptosis, and inhibition of metastatic tumor growth in vivo.[3,6–8] EZH2 has been reported to be overexpressed in a wide variety of human malignancies, including prostate, breast, endometrial, bladder, gastric, liver, esophageal, and pancreatic cancers.[5,9–15] EZH2 overexpression correlates with tumor aggression, metastasis, and poor prognosis.[5,9,12,14] Most recently, high EZH2 expression has been found to be associated with cancer aggressiveness and may serve as a prognostic marker in non-small cell lung cancer (NSCLC).[16]

Given the prominent role of EZH2 in cancer, there is much interest in understanding the molecular mechanism of EZH2 up-regulation in cancer. Transcriptionally, EZH2 expression has been shown to be repressed by tumor suppressor p53 and transcription factors E2F and E26 transformation-specific.[17–19] In addition, notably recent research shows that genomic loss of microRNA (miRNA)-101 results in overexpression of EZH2 in human tumor samples.[20] miRNAs are a class of short, highly conserved noncoding RNAs that regulate target genes through messenger RNA (mRNA) decay or translation repression.[21] Abnormalities of miRNA expression have been observed in various types of human cancers and are also related to prognosis and survival of patients with cancer.[22] Emerging data suggest that some miRNAs may function as oncogenes or tumor suppressor genes and play a critical role in cancer development.[23] Lung cancer is the

leading cause of cancer-related deaths in the world, and NSCLC accounts for approximately 80% of all cases. Despite many advances in diagnosis and treatment of NSCLC over the past several decades, the overall prognosis for patients with NSCLC remains poor. Therefore, there is an urgent need for novel therapeutic approaches for NSCLC. Because high EZH2 expression is closely correlated with tumor aggressiveness and poor prognosis in NSCLC,[16] identifying miRNA that potentially mediates EZH2 expression may provide new insights into the development of new therapeutic strategies for NSCLC. In this study, we show that miR-101, negatively regulating EZH2, inhibits NSCLC cell growth and invasion and improves cancer cells response to specific chemotherapy agents.

MATERIALS AND METHODS

Cell Lines and Tissue Samples

H226 (squamous), H358 (bronchioloalveolar adenocarcinoma), and A549 (adenocarcinoma) were provided by the American type culture collection. Human pulmonary giant cell carcinoma cell 801D was purchased from tumor cell bank of KeyGen Biotech (Nanging, China). All cells were maintained in RPMI-1640 containing 10% fetal bovine serum (FBS) with 100 IU/ml penicillin and 100 μg/ml streptomycin at 37°C with 5% CO_2. Paired NSCLC and adjacent nontumor lung tissue samples were collected at the time of surgery from 20 patients who underwent primary surgical resection of NSCLC with informed consent between December 2008 and March 2009 at Wuhan Union Hospital (Hubei, China). Tissue samples were fast frozen in liquid nitrogen after resection and stored at −80°C until use. All specimens were confirmed by the pathological examinations. No previous chemotherapy or radiotherapy was administered before resection. The clinical stage was defined according to American Joint Committee on Cancer Staging Manual.[24] This study was approved by the Human Research Ethics Committee of Wuhan Union Hospital.

Quantitative Reverse-Transcriptase Polymerase Chain Reaction Analysis of mRNA and miRNA Expression

Total RNA from tissues and the cultured cells was extracted using Trizol (Invitrogen, Carlsbad, CA) for both miRNA and mRNA analyses. For analysis of mature miR-101 expression, quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) was performed using Hairpin-it miRNAs qPCR Quantitation Kit (catalog number: QPM-010, GenePharma, Shanghai, China) according to the manufacturer's instructions. Relative expression was calculated by comparative CT method and normalized to the expression of U6 small RNA. EZH2-mRNA was quantified by qRT-PCR using Quantitect SYBR Green PCR Kit (Qiagen, Hilden, Germany) and normalized to β-actin. PCR primers were as follows: EZH2, 5′-CTTTTCTGTAGGCGATGT-3′ (forward) and 5′-ATAGTAAGTGCCAATGAGG-3′ (reverse) and β-actin, 5′-CAGAGCCTCGCCTTT GCC-3′ (forward) and 5′-GTCGC-CCACATAGGAATC-3′ (reverse). All qRT-PCRs were performed in triplicates.

Transfection of miRNA and Small Interfering RNA

The miR-101 mimics (catalog number: M-01-D) and small interfering RNA (siRNA) targeting EZH2 (siRNA-EZH2) (catalog number: A01025) were designed and synthesized by GenePharma (Shanghai, China). Cells at 50 to 60% confluency were transfected with 100 nmol/liter miR-101 mimics, 100 nmol/liter siRNA-EZH2, or their corresponding negative controls by Lipofectamine 2000 in Opti-Mem (Invitrogen). Six hours posttransfection, the culture medium was replaced with RPMI-1640 containing FBS. Forty-eight hours after transfection, cells were harvested for analysis. The transfection efficiency of miR-101 mimics or siRNA-EZH2 was confirmed by qRT-PCR detection of miR-101 or EZH2 mRNA expression (data not shown).

Luciferase Reporter Assay

Luciferase reporter vectors were constructed by cloning the wild type (wt) or mutant 3′-UTR of EZH2 mRNA (see Supplemental Digital content 1, http://links.lww.com/JTO/A55) into the XbaI-site of pGL3 vector (Promega, Madison, WI). A549 cells were transfected with wt or mutant reporter plasmid vector by Lipofectamine 2000 and then cotransfected with miR-101 mimics or negative control. After 36 hours, luciferase assays were performed using the dual luciferase assay system (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity for each sample.

Cell Proliferation Assay

Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) was used for estimating cell viability. In brief, cells were plated in 96-well plates at 5000 cells/well and incubated for 48 hours after transfection of 100 nM miR-101 mimics, 100 nM siRNA-EZH2, or their corresponding negative controls. At the end of incubation, the cell proliferation reagent WST-8 (10 μl) was added to each well and incubated for 3 hours at 37°C. Viable cell numbers were evaluated by measurement of optical density (OD) at 450 nm.

Cell Invasion Assay

Cell invasion was assessed using QCM 24-Well Collagen-Based Cell Invasion Assay (Chemicon, Billerica, MA) according to the manufacturer's protocol. In brief, 0.3 ml serum-free medium was added into each insert to rehydrate the collagen layer for 30 minutes, and then, it was replaced with 0.3 ml of prepared serum-free cell suspension containing 3.0×10^5 cells transfected with miR-101 mimics, siRNA-EZH2, or their corresponding negative controls; 0.5 ml medium containing 10% FBS was added to the lower chamber. After cells were incubated for 24 hours at 37°C, the noninvaded cells were removed with cotton swabs, and the invaded cells were stained with cell stain. The stained cells were dissolved in extraction buffer, and solutions were transferred to a 96-well culture plate for colorimetric reading of OD at 560 nm. The OD value represents cell invasion ability.

Anticancer Drug-Induced Apoptosis Assay

After 72 hours of transfection with miR-101 mimics, siRNA-EZH2, or their corresponding negative controls, H358

and H226 cells were treated with 40 nM paclitaxel (PTX) for 48 hours. Apoptosis was detected by using an annexin V-FITC kit (Bender Medsystems, Vienna, Austria). Briefly, cells were incubated with 5 μl of annexin V-FITC and 10 μl of propidium iodide in 200 μl binding buffer for 15 minutes at room temperature, immediately followed by flow cytometry. Data were analyzed by Cell Quest software. Cell lysates were prepared and subjected to Western blot analysis with antibodies specific for apoptosis-associated proteins.

Western Blot

Proteins from cells were extracted with lysis buffer (10 mmol/liter Tris-HCl, pH 7.4, 1% NP40, 0.1% deoxycholic acid, 0.1% sodium dodecyl sulfate (SDS), 150 mmol/liter NaCl, 1 mM ethylene diamine tetraacetic acid, and 1% Protease Inhibitor Cocktail) (Sigma, St. Louis, MO). Protein concentrations were determined by protein assay kit (Bio-Rad, Hercules, CA); 30 μg of protein was resolved by SDS-PAGE using a 10% polyacrylamide gel and transferred onto a nitrocellulose membrane. The membrane was incubated overnight at 4°C in blocking buffer (Tris-buffered saline, 0.1% Tween, and 5% nonfat dry milk) with the following primary antibodies: anti-EZH2 (Invitrogen), anti-H3K27me3 (Millipore, Billerica, MA), anti-(poly (ADP-ribose) polymerase) (PARP) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Bim (Gen-Script, Piscataway, NJ), and anti-β-actin (Cell Signaling Technology, Beverly, MA). A secondary antibody was incubated with the membrane for 1 hour after three washes with Tris-buffered saline and 0.1% Tween. Signals were detected with ECL detection reagent (Amersham, Piscataway, NJ).

Immunohistochemistry

Four-micrometer-thick formalin-fixed, paraffin-embedded tissue sections were deparaffined in xylene, rehydrated through graded alcohols, and processed using immunohistochemical SP method. Both negative and positive controls were set during staining, the former using phosphate-buffered saline to take the place of first antibody, whereas the latter using paraffin-embedded slice of prostate cancer known to be positive for EZH2 expression. Positive cells were defined as the presence of brown-yellow granules distributed in nucleus or cytoplasm, with stain intensity higher than the unspecific background. Percentage of positive cells was grouped as low (<20% of cells positive) or high (≥20% of cells positive).

Statistical Analysis

Statistical analysis was performed by SPSS software 13.0 (SPSS Inc., Chicago, IL). Values were presented as mean ± standard error of the mean. Differences/correlations between two groups were assessed by Student's t test, χ^2 test, and Pearson's correlation test. p less than 0.05 was considered to be statistically significant.

RESULTS

Decreased Expression of miR-101 is Associated with EZH2 Overexpression in NSCLC Tissues

The clinicopathologic characteristics in 20 patients with NSCLC are presented in Table 1. Total RNA was isolated from matched NSCLC and adjacent nontumor lung tissues, and the expression levels of miR-101 and EZH2 mRNA were determined by qRT-PCR. In 18 of 20 tumor samples, miR-101 expression was reduced in tumor tissues relative to matched nontumor tissues (Figure 1*A*). Furthermore, 16 tumor samples exhibited up to a twofold reduction in the level of miR-101. In contrast, EZH2 mRNA was overexpressed in 95% of tumor samples (19/20 samples) compared with the nontumor counterparts (Figure 1*B*). Then, we examined the association between miR-101 and EZH2 mRNA in these 20 resected tumor samples. A highly significant negative correlation was observed between miR-101 and EZH2 mRNA, reduced expression of miR-101 correlating with overexpression of EZH2 mRNA (Figure 1*C*). To better substantiate EZH2 overexpression in tumor tissues, we also conducted immunohistochemical analysis of paraffin-embedded tumor and nontumor samples from the same 20 patients. Representative examples of EZH2 immunostaining are displayed in Figure 1*D*. Tumor samples revealed intensive EZH2 staining; on the contrary, matched nontumor tissues displayed very weak staining. The result showed that the expression of EZH2 protein was similarly higher in tumor than in matched nontumor tissues (Table 2). Among these 20 tumor samples, 15 showed high-EZH2 protein expression and five showed low-EZH2 protein expression. We also found that EZH2 mRNA levels were significantly higher in tumor samples with high-EZH2 protein expression than in those with low-EZH2 protein expression ($p < 0.05$), which indicates that the increase in EZH2 mRNA expression is in keeping with the increase in EZH2 protein expression in tumor samples.

TABLE 1. The Clinicopathologic Data of Patients with NSCLC

Factors	
Age (yr)	61.4 ± 7.6
Gender (male/female)	12/8
TNM stage (I/II/III)	2/12/6
Histology (squamous/adenocarcinoma/others)	11/7/2

NSCLC, non-small cell lung cancer; TNM, tumor, node, and metastasis.

miR-101 Regulates EZH2 Expression by Binding the 3′-UTR of EZH2 mRNA

To explore whether miR-101 directly targets EZH2, we created luciferase reporter plasmids by cloning the wt or mutant 3′-untranslated regions (UTR) of EZH2 into pGL3 vector (Figure 2*A*). The wt or mutant reporter plasmid was cotransfected into A549 cells along with miR-101 mimics or the negative control. miR-101 mimics significantly suppressed the luciferase activity of the wt reporter plasmid in comparison with the negative control. Nevertheless, the luciferase activity of the mutant reporter plasmid was unaffected by simultaneous transfection with miR-101 mimics (Figure 2*B*), indicating that EZH2 is a direct target of miR-101. To further validate that EZH2 is negatively regulated by miR-101, we transfected miR-101 mimics into A549, H226, H358, and 801D cells and examined EZH2 mRNA and protein levels. The transfection efficiency was confirmed by

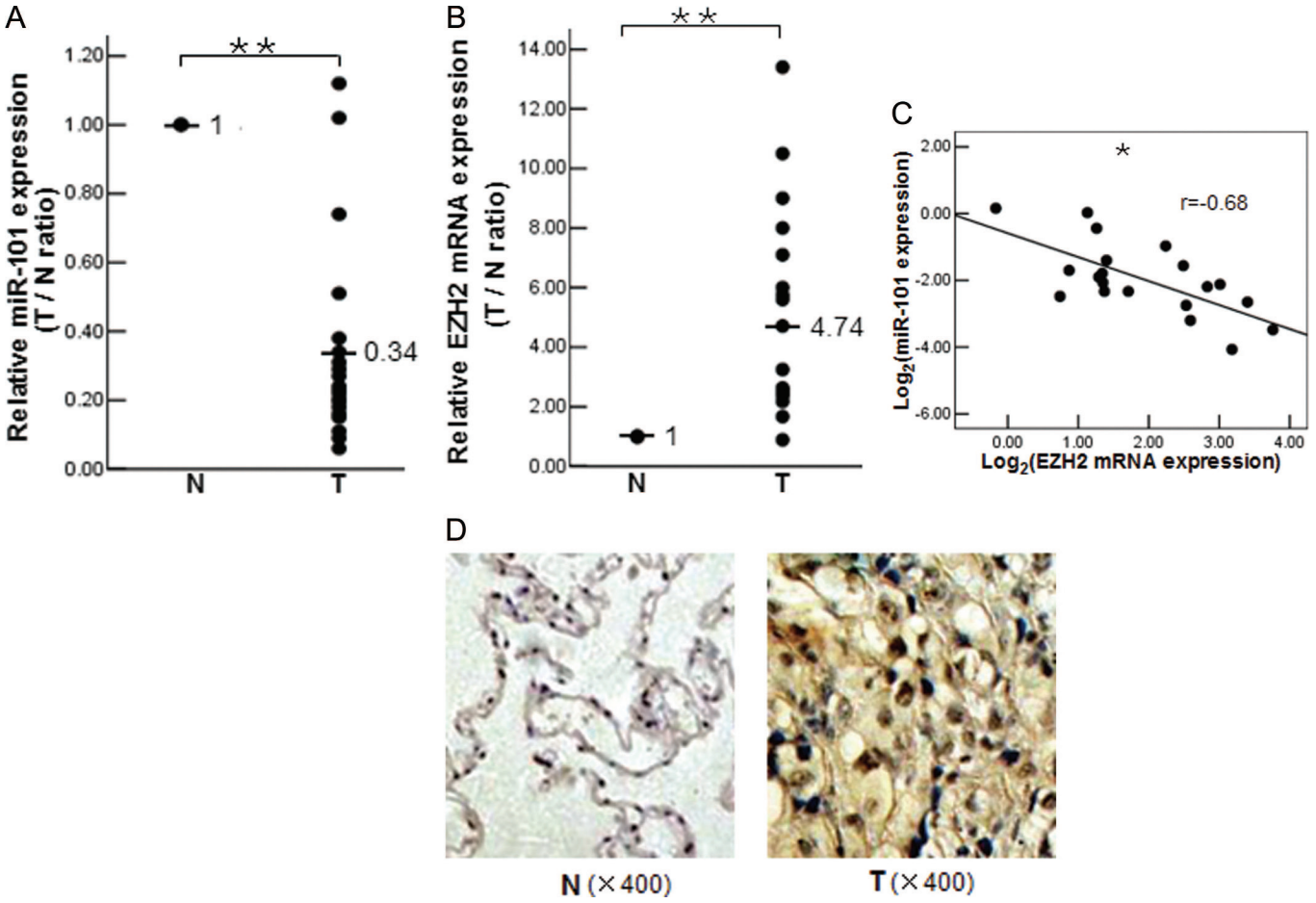

FIGURE 1. miR-101 and enhancer of zeste homolog 2 (EZH2) are inversely expressed in resected non-small cell lung cancer (NSCLC) tumors. Total RNA extracted from 20 pairs of matched NSCLC samples was used for quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) analysis of microRNA (miRNA) and messenger RNA (mRNA) expression. *A*, Relative expression of miR-101 in paired T and N. The results were normalized to U6 expression and expressed as fold change in tumor compared with matched N. *B*, Relative expression of EZH2 mRNA in paired T and N. The results were normalized to β-actin mRNA expression and expressed as fold change in tumor compared with matched N. *C*, Inverse correlation between EZH2 mRNA (data in panel *B*) and miR-101(data in panel *A*) in NSCLC tissues. *D*, Immunohistochemical staining patterns of EZH2 in matched NSCLC and adjacent N. T shows high-EZH2 expression (63.4% positive stained cells), and N shows low-EZH2 expression (7.6% positive stained cells). *$p < 0.05$; **$p < 0.001$; N, nontumor tissue; T, tumor tissue.

TABLE 2. Comparison of the EZH2 Protein Expression between NSCLC Tissues and Matched Nontumor Tissues

	No. of Cases	EZH2 Expression		
		High	Low	*p*
Tumor sample	20	15 (75%)	5 (25%)	<0.001
Matched nontumor sample	20	0 (0%)	20 (100%)	

Low, positive cells are <20%; high, positive cells are 20% or more; NSCLC, non-small cell lung cancer; EZH2, enhancer of zeste homolog 2.

qRT-PCR (see supplemental Figure S1A, Supplemental Digital Content 2, http://links.lww.com/JTO/A56). miR-101 overexpression led to a significant reduction of endogenous EZH2 mRNA (Figure 2*C*) and protein levels (Figures 2*D*, *E*) in NSCLC cell lines. As EZH2 mediates gene repression mainly by trimethylating H3K27 (H3K27me3), the levels of H3K27me3 were also evaluated at the same time. As shown in Figures 2*D*, *E*, overexpression of miR-101 in NSCLC cells concomitantly reduced expression of H3K27me3. These findings suggest that miR-101 may repress EZH2 expression through miR-101-binding sites at the 3′-UTR of EZH2.

Introduction of miR-101 or Specific Knockdown of EZH2 by siRNA Leads to Reduced Cell Proliferation and Invasion in NSCLC Cell Lines

To test whether miR-101 may exert tumor-suppressive functions by targeting EZH2, we investigated the effects of overexpression of miR-101 or knockdown of EZH2 on NSCLC cell proliferation and invasion. H226 and A549 cells were used for the proliferation assay, and 801D cells were used for the invasion assay because they displayed good ability to invade through the collagen-coated membranes.

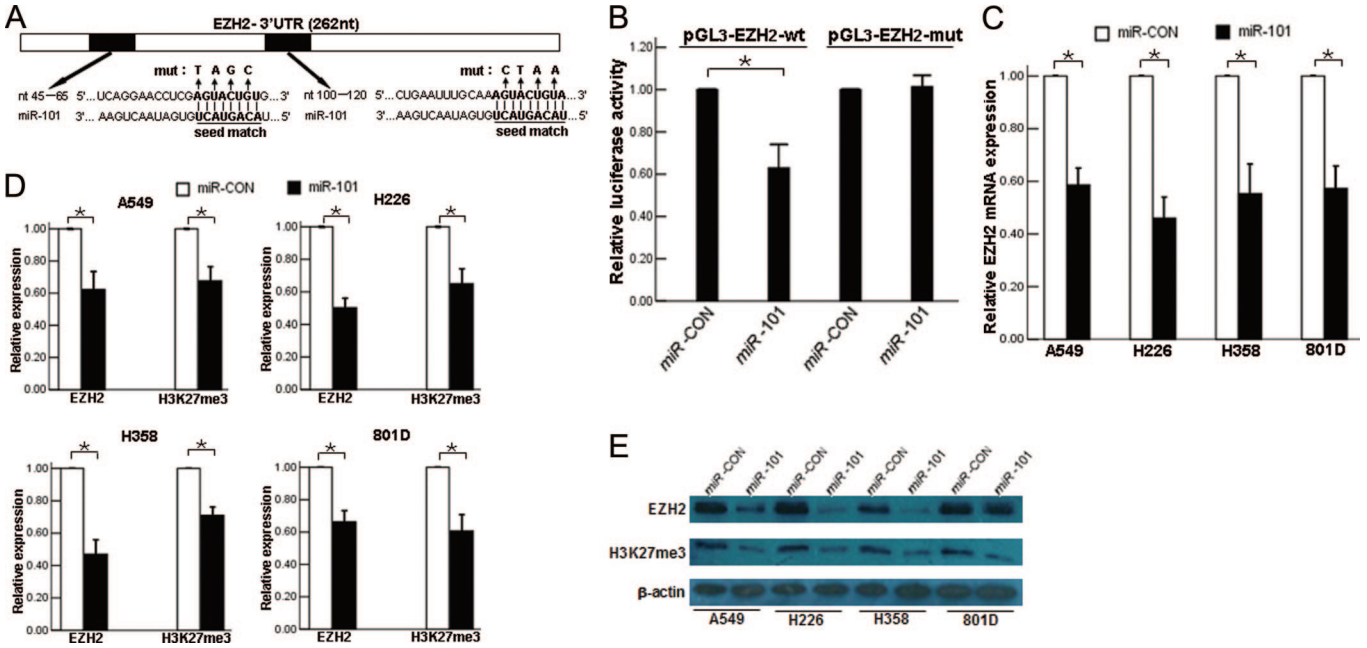

FIGURE 2. miR-101 suppresses enhancer of zeste homolog 2 (EZH2) expression by binding to the EZH2 3′-UTR. *A*, Schematic description of the EZH2 3′-UTR with putative binding sites for miR-101. The arrows indicate the mutant nucleotides. *B*, Luciferase reporter plasmids containing the wt or mutant EZH2 3′-UTR was cotransfected into A549 cells with miR-101 mimics or the negative control. After transfection for 36 hours, the luciferase activity was measured. The normalized luciferase activity in the control group was set to 1. miR-101 inhibited wt, but not mutant, EZH2 3′-UTR reporter activity. *C*, Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) analysis of EZH2 messenger RNA (mRNA) in cells transfected with miR-101 mimics or the negative control. The results were normalized to β-actin mRNA expression and expressed as fold change relative to the corresponding negative control. *D*, EZH2 and H3K27me3 protein levels in cells transfected with miR-101 mimics or the negative control were also detected by Western blot and standardized to β-actin protein. The results were expressed as fold change relative to the corresponding negative control. *E*, Representative gels display that miR-101 decreases expression of EZH2 and H3K27me3. All data are representative of three independent experiments. *$p < 0.05$; miR-CON, the negative control microRNA (miRNA).

H226, A549, and 801D cells were transfected with miR-101 mimics, siRNA-EZH2, or their corresponding negative controls. The transfection efficiency was verified by qRT-PCR (see supplemental Figure S1B, Supplemental Digital Content 2). The results showed that forced expression of miR-101 or siRNA-mediated EZH2 silencing significantly restrained the proliferation and invasion of NSCLC cells (Figures 3*A*, *B*).

miR-101 Overexpression or EZH2 Silencing Enhances the Apoptosis of NSCLC Cells in Response to PTX

It has been shown that miRNAs can affect cancer cells response to chemotherapy. The PTX is an anticancer drug widely used in the treatment of NSCLC. It is well established that PTX-induced cell kill is mainly mediated by apoptosis. Apoptosis resistance is a significant feature of cancer cells; therefore, we examined whether miR-101 can alter PTX-induced apoptosis of NSCLC cell lines. Among these four cell lines, H358 and H226 cells were insensitive to PTX treatment and, thus, were used for the apoptosis assay. We transfected H358 and H226 cells for 72 hours with miR-101 mimics or the corresponding negative control and then treated with 40 nM PTX for 48 hours. As a parallel control, we directly silenced EZH2 by transfection of siRNA-EZH2. All transfections were confirmed by qRT-PCR (see supplemental Figure S1C, Supplemental Digital Content 2). The percentage of apoptotic cells was evaluated by flow cytometric analysis of annexin V-FITC/PI-stained cells. As shown in Figures 4*A, B*, silencing of EZH2 or enforced expression of miR-101 sensitized cancer cells to PTX, resulting in a significant increase in apoptosis of NSCLC cells.

To clarify the potential mechanism of miR-101-mediated sensitization of NSCLC cells to PTX, we also detected the expression of a few apoptosis-associated proteins by Western blot at 48 hours of PTX treatment. EZH2 is known to inhibit the expression of the proapoptotic BH3-only protein Bim,[25] which is a tumor suppressor in epithelial solid tumors and which also is a determinant in PTX sensitivity.[26] So, the levels of Bim, EZH2, and cleaved PARP were measured. As expected, in miR-101 or siRNA-EZH2-transfected cells, the level of EZH2 was lower than the corresponding control cells; accordingly, both Bim and cleaved PARP levels were higher in miR-101 or siRNA-EZH2-transfected cells compared with the corresponding control cells (Figures 4*C*, *D*).

DISCUSSION

Although miRNA expression signatures in many types of human malignancies have been identified, elucidation of

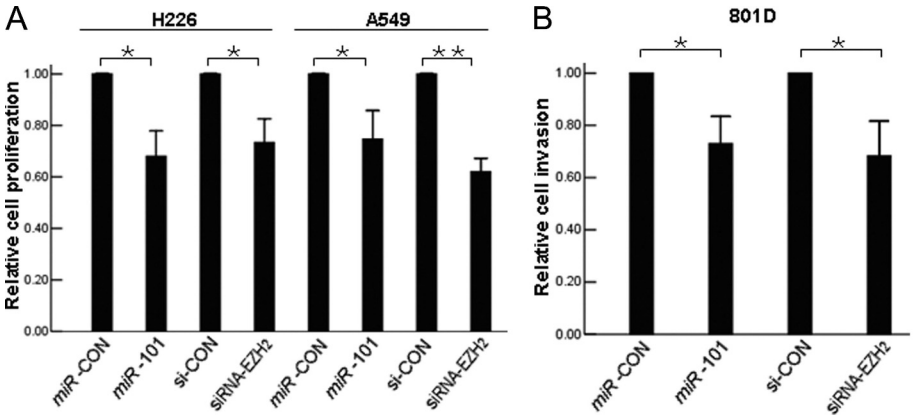

FIGURE 3. miR-101 overexpression or enhancer of zeste homolog 2 (EZH2) knockdown inhibits non-small cell lung cancer (NSCLC) cell proliferation and invasion. Cells transfected with 100 nmol/liter miR-101 mimics or small interfering RNA (siRNA)-EZH2, compared with the corresponding negative controls, were used for the proliferation and invasion assay. *A,* Cell proliferation was analyzed by CCK-8 assay in H226 and A549 cells. The results were expressed as fold change relative to the corresponding negative controls. *B,* Cell invasion was determined in 801D cells using Chemicon cell invasion assay. The results were expressed as fold change relative to the corresponding negative controls. All data are representative of three independent experiments. *$p < 0.05$; **$p < 0.001$; miR-CON, the negative control microRNA (miRNA); si-CON, the negative control siRNA.

the role of the dysregulation of specific miRNAs in carcinogenesis remains in the initial stage of development. In this study, we show that miR-101 is underexpressed in NSCLC tumor tissues and that it suppresses EZH2 expression by binding to 3′-UTR of EZH2 in NSCLC cells. Moreover, the current report demonstrates that miR-101 restrains NSCLC cell proliferation and invasion and sensitizes cancer cells to PTX-induced apoptosis in vitro by suppression of EZH2 expression.

EZH2 is frequently overexpressed in a wide range of tumor types and plays a crucial role in tumorigenesis process. EZH2 has been shown to promote oncogenic transformation and increase the biologic aggressiveness of cancer cells through the epigenetic silencing of target genes. Our study revealed that EZH2 mRNA and protein expression were significantly higher in NSCLC tumor tissues than in matched nontumor tissues (Figures 1*B*, *D*). Although relatively little is known about how EZH2 becomes overexpressed in NSCLC, one such mechanism is through regulation by miRNAs. Human miRNA genes are frequently located at fragile sites and cancer-associated genomic regions.[27] It has been reported that abnormal down-regulation of miR-101 could lead to EZH2 overexpression in prostate and bladder cancer.[20,28] In this study, miR-101 was down-regulated in NSCLC tumor samples compared with matched nontumor counterparts, which was consistent with previous studies.[29,30] Furthermore, we observed a highly significant negative correlation between miR-101 and EZH2 mRNA in tumor samples (Figure 1*C*). miR-101, a miRNA commonly down-regulated in cancers, has been shown to be implicated in cancer-related processes such as cell growth, invasion, and apoptosis.[20,28,31–34] Therefore, we postulated that miR-101 regulated EZH2 expression in NSCLC.

This postulation was further confirmed by subsequent experimental studies on NSCLC cell lines. As shown in Figure 2*B*, the activity of the luciferase reporter with wt but not mutant EZH2 3′-UTR was significantly inhibited in A549 cells transfected with miR-101 mimics compared with the negative control. In addition, transfection of NSCLC cells with miR-101 mimics caused a dramatic decrease in EZH2 mRNA and protein levels and concomitantly reduced H3K27me3 expression. These findings provide strong evidence that miR-101 down-regulates EZH2 expression by directly targeting the 3′-UTR of EZH2 mRNA. Although we prove that EZH2 is a direct target of miR-101 in NSCLC, it is still unknown whether miR-101 may exert tumor-suppressive functions in NSCLC. Accordingly, we investigated the effect of miR-101 on the biological behavior of NSCLC cells in vitro. We observed that overexpression of miR-101 inhibited the proliferation and invasion of NSCLC cells. Moreover, knockdown of EZH2 by siRNA in NSCLC cells also resulted in reduced cell proliferation and invasion. These data indicate that suppression of NSCLC cell proliferation and invasion by miR-101 may be mediated, at least in part, through down-regulation of EZH2.

The treatment of many malignancies, including NSCLC, is usually impeded by the intrinsic or acquired resistance to the anticancer drugs used. Deregulated apoptosis is a common cause of cancer therapy chemoresistance because the apoptosis-inducing ability of anticancer agents may at least partly account for drug efficacy. NSCLC is characterized by its poor survival and resistance to chemotherapy-induced apoptosis both in vitro and in vivo.[35–37] PTX, which is extensively used to treat NSCLC, kills tumor cells chiefly through inducing apoptosis. Therefore, the role of miR-101 in regulating PTX-induced apoptosis in NSCLC cells deserves investigation. In this study, miR-101 overexpression or siRNA-mediated EZH2 depletion in NSCLC cells stimulated PTX-induced apoptosis and led to a decrease in EZH2 protein and an increase in Bim protein. Previous studies demonstrated that enforced expression of Bim increased the susceptibility of NSCLC cells to PTX-mediated killing, and knock-

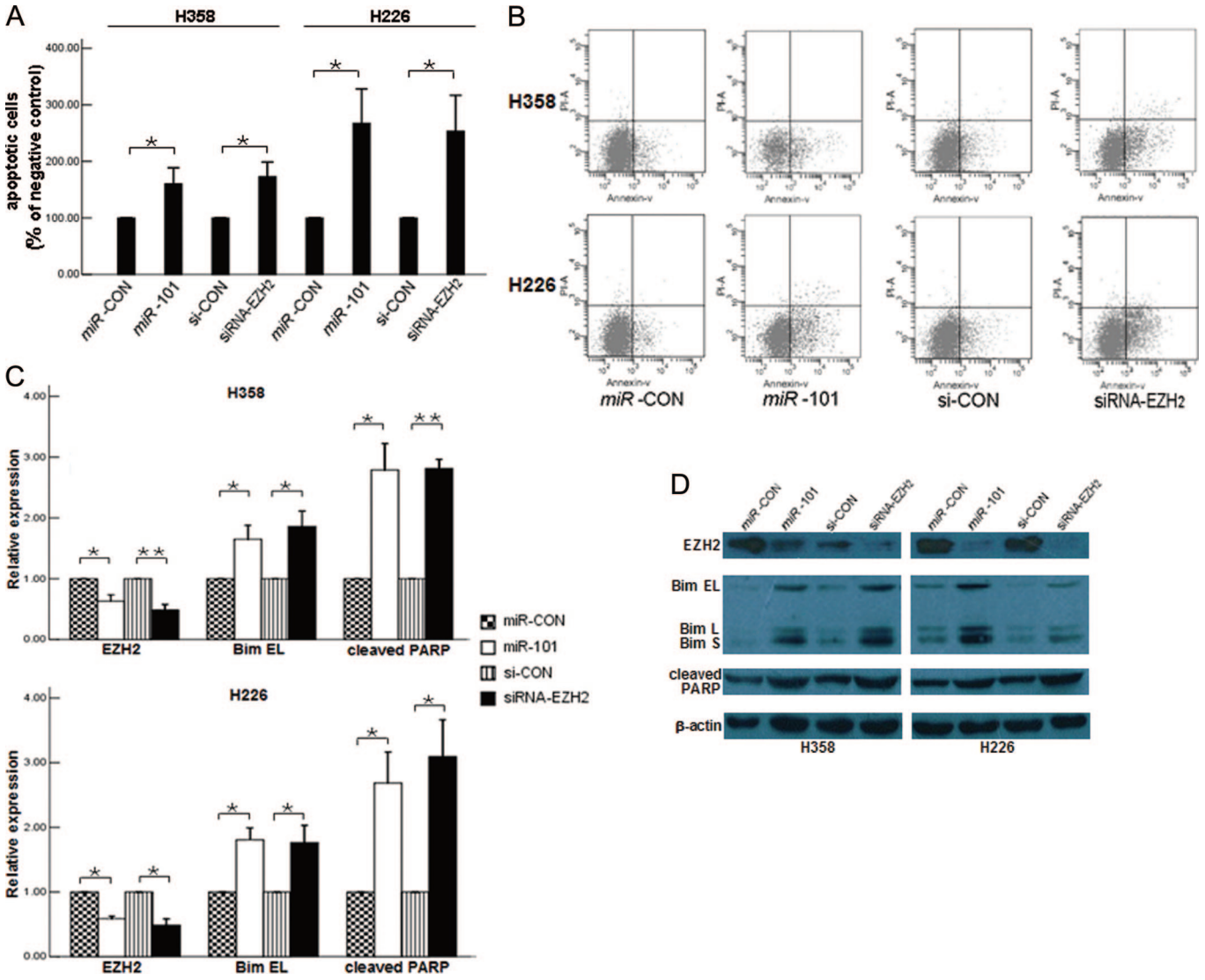

FIGURE 4. Overexpression of miR-101 or silencing of enhancer of zeste homolog 2 (EZH2) sensitizes non-small cell lung cancer (NSCLC) cells to PTX-induced apoptosis. H358 and H226 cells transfected for 72 hours with miR-101 mimics, small interfering RNA (siRNA)-EZH2, or their corresponding negative controls, and then treated with 40 nM PTX for 48 hours, were harvested for apoptosis analysis. *A*, Apoptosis was measured by flow cytometry for annexin V-FITC/PI double staining. The results were expressed as percentages of the corresponding negative controls. *B*, Representative cytograms show that miR-101 overexpression or EZH2 depletion augments PTX-induced apoptosis. *C*, Cell lysates were prepared for Western blot analysis of EZH2, Bim, and cleaved PARP expression. The results were normalized to β-actin protein expression and expressed as fold change relative to the corresponding negative controls. *D*, Representative immunoblots show that EZH2 suppression by miR-101 or siRNA-EZH2 induces Bim and cleaved PARP expression. All data are representative of three independent experiments. *$p < 0.05$; **$p < 0.001$; miR-CON, the negative control microRNA (miRNA); si-CON, the negative control siRNA.

down of Bim reduced PTX-mediated killing of NSCLC cells, suggesting that Bim is an important molecular link between PTX and apoptosis.[38] EZH2 has been shown to regulate apoptosis through epigenetically modulating Bim expression.[25] Together with these previous research achievements, our results indicate that EZH2 repression by miR-101 induces Bim expression and, thus, enhances PTX-induced apoptosis in NSCLC cells. Certainly, as a specific miRNA has the potential to target multiple genes, miR-101 may function through cooperative regulation of its other target genes. Nevertheless, other targets of miR-101 besides EZH2 still need to be identified in NSCLC.

In conclusion, miR-101, which is down-regulated in NSCLC tumor tissues, inhibits cellular proliferation and invasion and increases apoptosis induced by PTX in NSCLC cell lines. These effects are at least partially due to direct suppression of EZH2 by miR-101. Our findings imply that therapeutic strategies aimed at restoration of miR-101 expression or depletion of EZH2 may be beneficial to patients with NSCLC.

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