The expression and significance of the enhancer of zeste homolog 2 in lung adenocarcinoma

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Abstract. Lung adenocarcinoma, with increased incidence in the world, exhibits poor prognosis and is usually resistant to conventional chemotherapy due to drug resistance. The enhancer of zeste homologue 2 (EZH2), a histone methyltransferase, plays a key role in tumorigenesis and cancer development through chromatin remodeling in various types of cancer. However, its potential role in lung adenocarcinoma has not been well defined. In this study, the expression of EZH2 was examined in lung adenocarcinoma tissues and cell lines. Most interestingly, EZH2 overexpression was detected in tumor tissue and significantly correlated with histological differentiation, pathological tumor-node-metastasis stage and smoking history, but not with gender or age. Furthermore, EZH2 overexpression was also detected in cisplatin-resistant cancer cells rather than cisplatin-sensitive cells. Short hairpin RNA targeted against EZH2 inhibited cell proliferation and migration, and led to G(2)/M cell cycle arrest and apoptosis in both cisplatin-resistant and cisplatin-sensitive cell lines. Moreover, EZH2 knockdown enhanced cisplatin sensitivity of cisplatin-resistant cells and reduced the expression levels of multidrug resistance-related protein 1. Our study suggests that EZH2 contributes to the progression of lung adenocarcinoma, and the deletion of EZH2 inhibits cancer and resensitizes cells to cisplatin in lung adenocarcinoma.

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Introduction

Lung cancer (LC) is a leading cause of cancer-mortality worldwide. Current medical therapy is relatively effective, but the 5-year survival rate is only 14% (1). Lung adenocarcinoma (LA) is one major type of LC and accounts for 40% of non-small cell LC (NSCLC) (2). With increased smoking and air pollution, the incidence of LA is increasing in the world (3) including China (4). Moreover, LA usually responds poorly to chemotherapy (5) and most patients of LA are resistant to cisplatin (DDP). Therefore, better defining the pathogenesis of LA and exploring novel therapeutic targets sensitive to chemotherapy are urgently required.

The gene of enhancer of zeste homolog 2 (EZH2), encoding a polycombgroup (PcG) protein, is thought to have the potential to silence genes involved in tumorigenesis. Overexpression of EZH2 has been reported in metastatic prostate cancer and breast cancer, and considered to have diagnostic and prognostic value (6-8). Recent studies indicate that EZH2 is a key component of the polycomb-repressive complex required for maintenance of a stem cell state, and overexpression of EZH2 is involved in drug resistance in ovarian cancers (9). However, the role of EZH2 in cell proliferation, migration, cell cycle regulation and apoptosis remains unclear in LA.

The development of multidrug resistance (MDR) to cancer chemotherapy is a major obstacle to the medical treatment of LA. Multidrug resistance 1 (MDR1), multidrug resistance-related protein 1 (MRP1) and lung resistance protein (LRP) are thought to be associated with the development of MDR (10-12). However, the relationship between the effect of EZH2 and the development of MDR to LA chemotherapy is still unknown.

In our study, we investigated the EZH2 expression in LA specimens, corresponding distant normal tissues and LA cell lines including cisplatin-resistant cell line (A549/DDP) and cisplatin-sensitive cell line (A549), and studied its correlation with the clinicopathological features. Furthermore, we investigated the effects of EZH2 depletion on cell proliferation, migration, cycle control and apoptosis regulation in LA cell lines *in vitro*. We used EZH2 short hairpin RNA (shRNA) to silence EZH2 gene and further

Table I. Primers used for the RT-PCR.

Gene	Sequence of primer	Length (bp)
EZH2	F: 5'-GCGGGACTAGGGAGTGTTC-3' R: 5'-AGCAACTGCATTCAGAGTCTT-3'	83
β-actin	F: 5'-GACAGGATGCAGAAGGAGATTACT-3' R: 5'-TGATCCACATCTGCTGGAAGGT-3'	141
MDR1	F: 5'-CCCATCATTGCAATAGCAGG-3' R: 5'-GTTCAAACTTCTGCTCCTGA-3'	157
MRP1	F: 5'-AGTTCTGCGGTGCTGTTGTG-3 R: 5'-TTCGCTGAGTTCCTGCGTAC-3'	218
LRP	F: 5'-CATCATTCGCACTGCTGTCT-3' R: 5'-TTTCTCGGCTTCTGACTGGT-3'	257

analyzed the effects of EZH2 knockdown on acquired cisplatin resistance.

Materials and methods

Human tissues and clinical data. Neoplastic tissues and distant normal tissues (>5 cm from the margin of the tumor) were obtained from 69 patients (age, range 41-73 years; gender, 36 men and 33 women) who underwent a surgical resection of LA at Qilu Hospital, Shandong University from the year 2004 to 2009. The samples were divided into two halves, one half was fixed in formalin and embedded with paraffin and the other half was stored at -80˚C until use. After histological examination, tumor samples were classified as well differentiated (n=22), moderately differentiated (n=28) and poorly differentiated (n=19). The pathological stage (pStage) was made based on American Joint Committee on Cancer guidelines for postoperative tumor-node-metastasis (TNM) classification. Written informed consent was obtained from each patient. This study was approved by the Medical Ethics Committee of the Qilu Hospital, Shandong University.

Immunohistochemistry staining. Specimens were deparaffinized and rehydrated. After antigen retrieval, the primary antibody against EZH2 mAb ab3748 (Abcam, USA) was used at dilution of 1:1000 and incubated overnight at 4˚C. The second antibody from SP reagent kit (Zhongshan Biotechnology Co., Beijing, China) were employed to visualize antibody binding and stained with DAB. The stained slides were reviewed and scored independently by two pathologists who had no knowledge of patient information, and both observers re-examined the immunostained slides with discrepant scores to determine a consensus score. Expression of EZH2 was scored semiquantitatively by the combination of intensity and proportion of the positively stained tumor cells as follows: negative expression, no staining; 1+, <5% tumor cells staining; 2+, 5-25% tumor cells staining; 3+, 26-50% tumor cells staining; and 4+, >50% tumor cells staining.

Cell culture, EZH2 shRNA transfection, cell migration, and cell proliferation. The study included human lung adenocarcinoma cell lines A549 and A549/DDP. A549 cell lines (CCL-185) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured according to the protocol provided by the ATCC. A549/DDP cell line, resistant to cisplatin, was generously provided by the Beijing Cancer Institute (Beijing, China). The A549/DDP clones showed 9-fold greater drug resistance to cisplatin than A549 cells and it was cultured with 6 nM cisplatin (Sigma) to maintain the drug resistance phenotype.

The sequence of EZH2 mRNA from 461-481nt was selected for RNAi target, and the shRNA plasmid for EZH2 gene was constructed according to the protocol of pSuper RNAi System™ from the OligoEngine Co. A549 cell was transfected with pSuper-shEZH2 vectoror empty pSuper vector as the control using Lipofactamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Transfectants were screened with hygromycin B (Roche, Indianapolis, IN, USA).

For proliferation assay, cells were seeded in a 96-well plate with 200 μl medium. 3-(4,5)-dimethylthiazol(-2-y1)-3,5-diphenyl tetrazolium bromide (MTT, Sigma) was added at concentration of 0.5 mg/ml and then incubated for 4 h at 37˚C. The medium was removed, and dimethyl sulfoxide (DMSO, Sigma) was added to each well to dissolve crystals by shaking the plate gently for 30 min in the dark. The OD value in each well was determined at 570 nm by a Microplate reader (Thermo Varioskan Flash).

The migration assay was performed using the transwell system (24-wells, 8 μm pore size with polycarbonate membrane, BD Biosciences, San Jose, CA, USA) and followed the BD protocol. Cells were stained with DAPI (Beyotime Biotechnology, China). DAPI is known to form fluorescent complexes with natural double-stranded DNA in nuclear, showing a blue fluorescence. The total number of cells invading and adhering to the lower surface was counted in 6 representative fields under an Olympus light microscope.

RNA isolation and reverse transcription PCR. RNA isolation and RT-PCR was performed as previously described (13). Total RNA was extracted from LA cell lines using TRIzol reagent (Invitrogen) based on the suggested protocol.

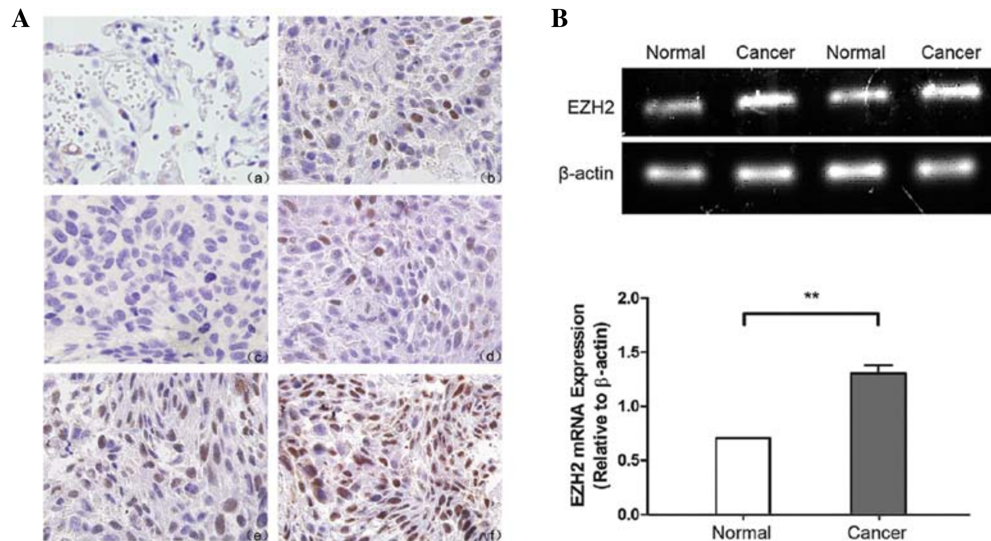

Figure 1. EZH2 expression in LA specimens and normal tissues were analyzed. (A) Immunohistochemical staining of EZH2 expression in normal and cancerous lung tissues. A representative is shown of normal, and cancer (negative and positive expression) tissues. a, normal tissue (negative expression); b, cancerous tissue (positive expression); c, pStage I tumor tissue (negative expression); d, pStage I tumor tissue (weak expression); e, pStage II tumor tissue (moderate expression); f, pStage III tumor tissue (strong expression). (B) RT-PCR analyses of EZH2 mRNA in normal and cancerous lung tissues. A representative result is shown. The relative quantitation of EZH2 mRNA ***P<0.001 and **P<0.01 vs. normal tissues. Data are compiled from 3 independent experiments in each condition.

Table II. The expression of EZH2 in lung adenocarcinoma and distant normal tissues.

		EZH2 expression		
Variables	n	Positive	Negative	P-value
Distant normal tissues	69	8	61	<0.01
Lung adenocarcinoma	69	44	25	

The mRNA was reverse-transcribed to cDNA by using the PrimeScript RT-PCR kit (Takara). The primers used for the RT-PCR are shown in Table I. PCR products were separated on a 2% agarose gel and viewed by ethidium bromide (EB; Molecular Probes, Eugene, OR, USA) staining. The data were analyzed using AlphaImager 2200 software (Alpha Innotech, San Leandro, CA, USA).

Western blot analysis. Western blot analysis was performed as previously described (14). Cells were lysed with RIPA lysis buffer (Beyotime Biotechnology). For protein assay, BCA protein assay kit (Beyotime Biotechnology) was used. Protein samples were electrophoresed on 12% SDS-PAGE and then electroblotted onto polyvinylidene fluoride (PVDF, Millipore) membranes. Immunoblotting was performed using the following antibodies: EZH2 antibody (ab3748) (1:1000, Abcam, USA) or monoclonal anti-β-actin antibody (1:4000, A1978, Sigma). Membranes were further incubated with polyclonal goat anti-mouse HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at room temperature. Bands were detected by an ECL kit (Cell Signaling). The intensity of each band was quantified using ImageJ 1.32 software (National Institutes of Health, Bethesda, MD, USA).

Flow cytometry. All fluorescence-conjugated antibodies used in this study were purchased from BD Bioscience. Trypsin-treated cells were washed with PBS and fixed in 70% ethanol overnight. Cells were then washed with PBS again and stained with propidium iodide (PI) (50 μg/ml) solution for cell cycle analysis. Cells were Annexin V-FITC and PI treated as recommended by the supplier. Cells were analyzed by flow cytometry using a flow cytometer (BD FACSCalibur, MI). To analyze intracellular proteins, cells were first fixed and permeabilized, and then stained with appropriate mAbs using a Cytofix/Cytoperm plus kit (eBioscience, San Diego, CA, USA). Flow cytometric analysis was performed using FlowJo software (Tree Star Inc., San Carlos, CA, USA).

Statistical analysis. Data are presented as the mean ± SD, SPSS software package (versions 13.0, SPSS, Inc., Chicago, IL, USA) was used for all statistical analysis. Correlations of EZH2 expression with clinicopathological factors were examined by χ^2 tests. Other data from experiments were analyzed by Student's t-test or ANOVA wherever appropriate. Difference was considered significant when the P-value was <0.05.

Results

EZH2 overexpression in LA tissues. In our study, EZH2 was detected as nuclear staining of the cells. EZH2 expression was detected in 44 (63.8%) tumor tissues, including 12 cases (27.2%) with weak expression (1+), 18 cases (40.9%) with moderate expression (2+) and 14 cases (31.8%) with strong expression (3+-4+). However, EZH2 expression was only detected in 8 (11.6%) corresponding distant normal tissues (Fig. 1A; Table II).

In addition, we measured EZH2 mRNA expression with RT-PCR. It turned out to be consistent with those observed by immunohistochemistry (Fig. 1B).

Table III. Relation between expression of EZH2 and clinicopathological characteristics of patients with surgically resected lung adenocarcinoma.

Variables	No. of cases	EZH2 expression		P-value
		Negative	Positive	
Gender				0.601
Male	36	12	24	
Female	33	13	20	
Age (years)				0.532
≤59	27	11	16	
>59	42	14	28	
Tobacco history				0.037
Smoker	39	10	29	
Never smoke	30	15	15	
TNM stages				0.01
I	22	15	7	
II	26	5	21	
III	21	6	15	
Pathology grade				0.036
Well	22	12	10	
Moderate	28	10	18	
Poor	19	3	16	

Moreover, the association of EZH2 expression with clinicopathological parameters was further analyzed. It showed that the EZH2 expression was significantly correlated with tobacco history (P=0.015), TNM stages (P=0.01), and pathology grade (P=0.036) (Table III), which indicated that the EZH2 was involved in tumor development.

Elevation of EZH2 expression in drug-resistant LA cancer cells. To investigate the role of EZH2 in drug resistance, we measured EZH2 expression in a cisplatin-resistant LA cell line (A549/DDP) and a cisplatin-sensitive cell line (A549). Enhanced expression of EZH2 mRNA was observed in A549/DDP cells compared with that in A549 cells (Fig. 2A). These findings were further confirmed by western blot analysis (Fig. 2B).

Suppression of EZH2 inhibited LA cell proliferation and migration. To understand the role of EZH2 in cell proliferation and migration, we knocked down EZH2 gene expression by shRNA interference. An obvious decrease of EZH2 mRNA level was observed in EZH2-knockdown cells (Fig. 3A).

Transfection of EZH2-targeting construct (shEZH2) into cells resulted in lower proliferation both in A549 and A549/DDP compared with controls (Fig. 3B). Meanwhile, decreased migration was also observed both in A549 and A549/DDP cells after shEZH2 transfection (Fig. 4).

Suppression of EZH2 causes LA cell cycle arrest and apoptosis acceleration. Flow cytometry was used to assay cell cycle and apoptosis, we found that transfection of shEZH2 into A549 and A549/DDP cells led to G(2)/M cell cycle arrest (Fig. 5A) and increased apoptosis in EZH2-depleted cancer cells (Fig. 5B).

Suppression of EZH2 sensitized LA cells to cisplatin and decreased MRP1 expression. To investigate the effect of EZH2 depletion on cisplatin chemoresistance, MTT assays were performed. Cell survival was significantly inhibited after DDP treatment in A549/DDP-shEZH2 cells compared to control

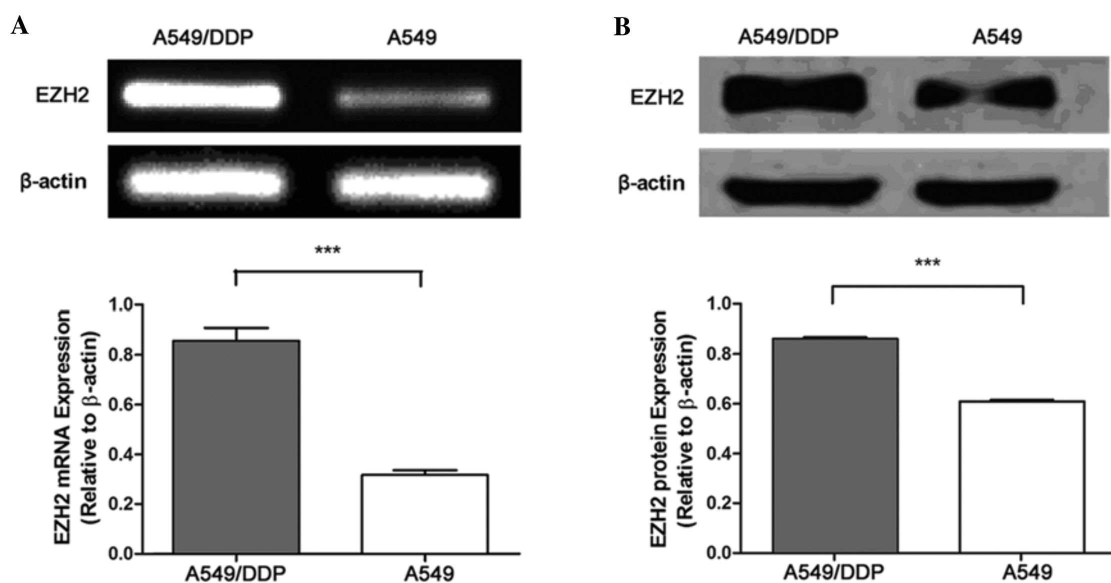

Figure 2. EZH2 expression in A549/DDP and A549 cell lines were analyzed. (A) The mRNA level of EZH2 in A549/DDP and A549 cell lines were analyzed. The relative level of EZH2 mRNA was upregulated in A549/DDP cells compared with A549 cells by RT-PCR. Data represent the mean and the standard deviation from three independent experiments. ***P<0.001 vs. A549 cells. (B) The protein level of EZH2 in A549/DDP and A549 cell lines were analyzed. The level of EZH2 protein was upregulated in A549/DDP cells compared with A549 cells by western blot analysis. Data represent the mean and the standard deviation from three independent experiments. ***P<0.001 vs. A549 cells.

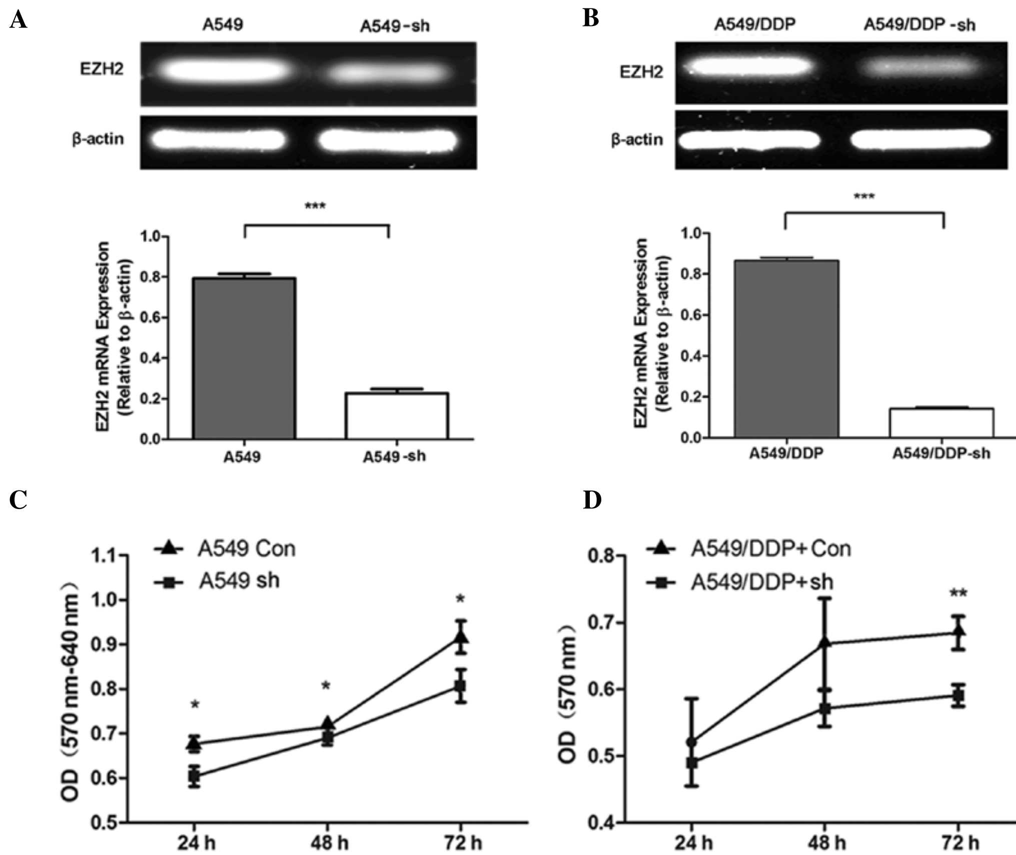

Figure 3. The stable transfection of EZH2-shRNA and the effect of EZH2 depletion on cell proliferation in A549 and A549/DDP cells were analyzed. (A) The EZH2-shRNA stable transfection in A549 cell line. Expression of EZH2 mRNA was decreased in shEZH2-A549 cells compared with control A549 cells by RT-PCR. Data represent the mean and the standard deviation from three independent experiments. ***P<0.001 vs. untransfected cells. (B) The EZH2-shRNA stable transfection in A549/DDP cell line. Expression of EZH2 mRNA was decreased in shEZH2-A549/DDP cells compared with control A549/DDP cells by RT-PCR. Data represent the mean and the standard deviation from three independent experiments. ***P<0.001 vs. untransfected cells. (C) The effect of EZH2 depletion on cell proliferation in A549 cells. EZH2 depletion in A549 cells significantly inhibited cell proliferation when compared with control cells. *P<0.05 vs. control cells. (D) The effect of EZH2 depletion on cell proliferation in A549/DDP cells. EZH2 depletion in A549/DDP cells significantly inhibited cell proliferation on 72 h when compared with control cells. **P<0.01 vs. control cells.

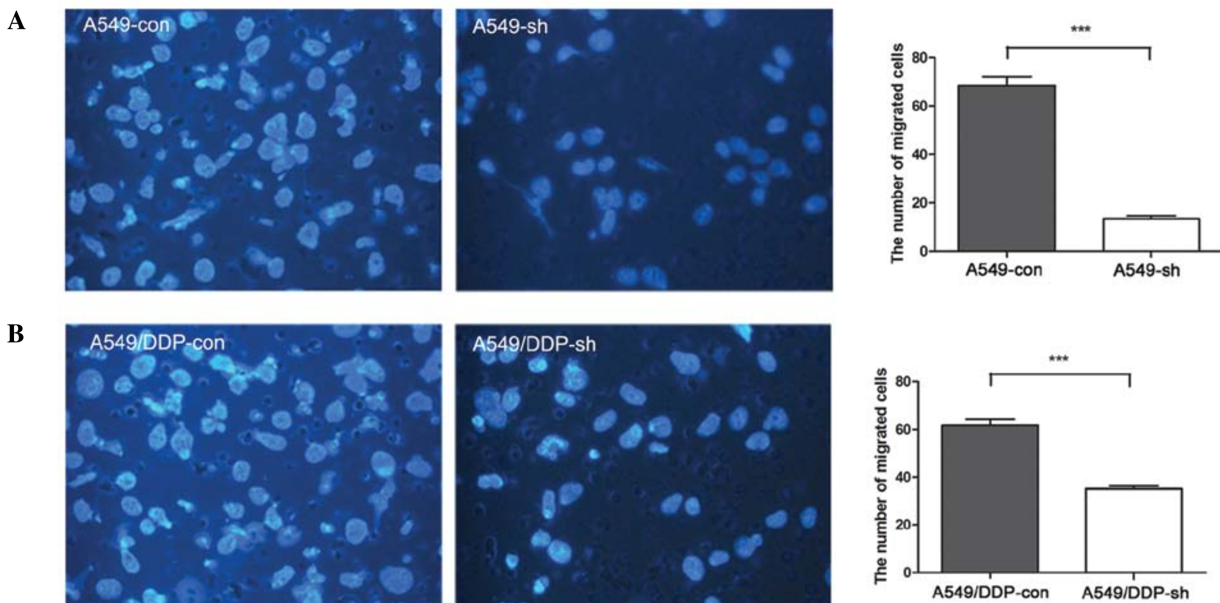

Figure 4. The effect of EZH2 depletion on cell migration in A549 and A549/DDP cells were analyzed. (A) The effect of EZH2 depletion on cell migration in A549 cells. Cells invading the polycarbonate membrance of transwell chamber was 87.5±3.3 and 23.5±5.1 per high power field in A549-con and A549-sh group, respectively (***P<0.001). (B) The effect of EZH2 depletion on cell migration in A549/DDP cells. Cells invading the polycarbonate membrane of transwell chamber was 90.2±4.3 and 25.1±4.5 per high power field in A549/DDP-con and A549/DDP-sh group, respectively (***P<0.001).

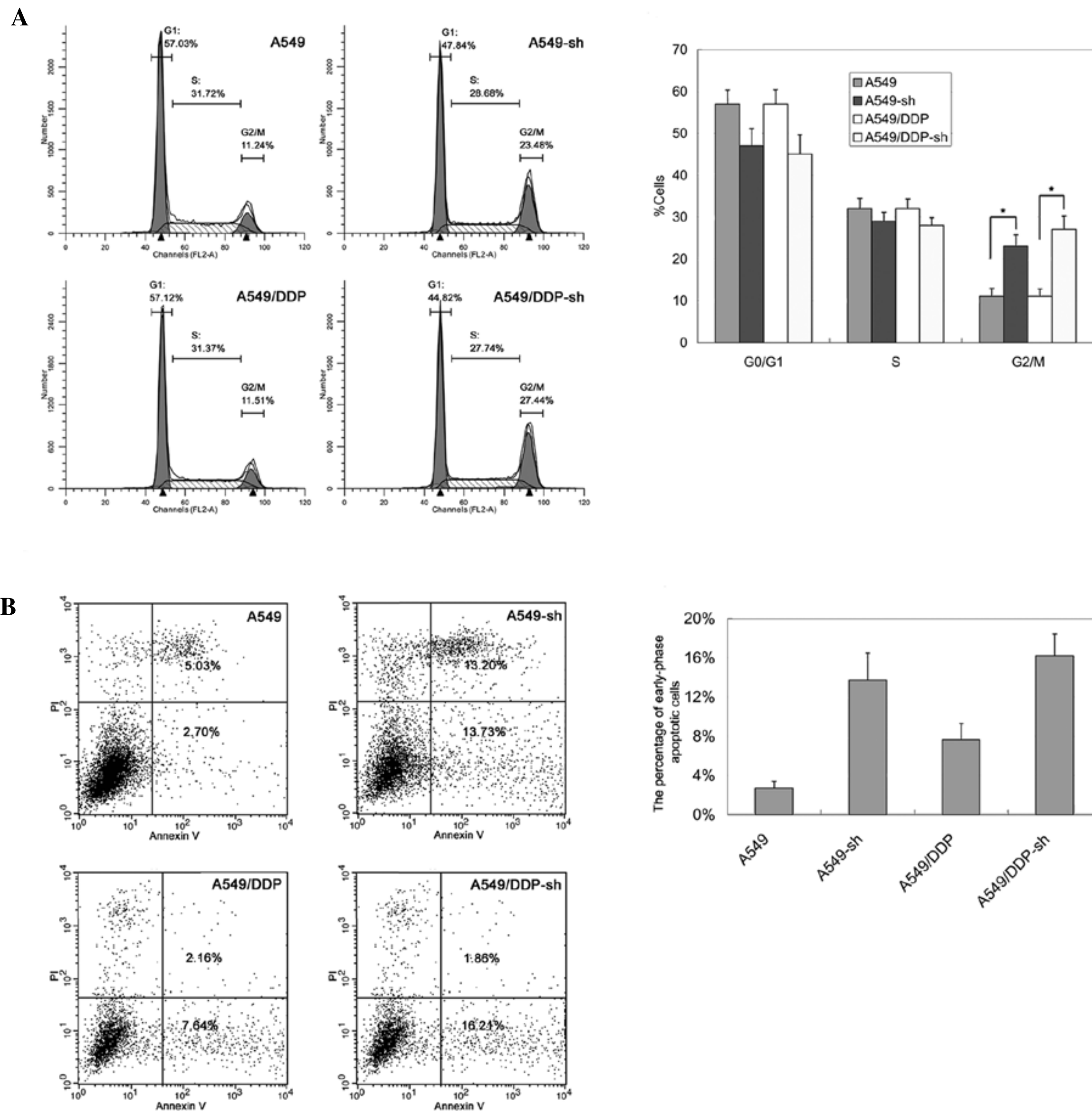

Figure 5. The effect of EZH2 depletion on cell cycle and apoptosis in A549 and A549/DDP cells were analyzed. (A) Effect of EZH2 depletion on cell cycle in A549 and A549/DDP cells. The DNA content was analysis by flow cytometry. Samples were fixed and stained with PI. Histograms show cell cycle distribution. (B) Effect of EZH2 depletion on cell apoptosis in A549 and A549/DDP cells. The percentage of apoptotic cells was determined by Annexin V-FITC/PI staining. Data were compiled from 3 independent experiments in each condition.

cells. The cisplatin IC_{50} was lower in A549/DDP-shEZH2 (90.400 μM) cell vs. control cells (131.2 μM). EZH2 deletion sensitized cancer cells to cisplatin, leading to a significant decrease of IC_{50} (Fig. 6A).

Furthermore, to study the possible mechanism of EZH2 in drug resistance, we measured mRNA level of MDR1, MRP1, and LRP expression. We found EZH2 deletion led to a down-regulation of MRP1 in A549/DDP-shEZH2 cells (Fig. 6B). EZH2 deletion did not have significant influence on the expression of MDR1 and LRP (data not shown).

Discussion

In the current study, we found that EZH2 expression was increased in tumor tissues compared to normal tissues. EZH2 expression was related to tumor pTNM stage. To investigate the potential role of EZH2 in LA, we knocked down EZH2 in LA cells with shRNA, and found that silencing of EZH2 gene inhibited cell proliferation, migration, arrested G2/M cell cycle and increased apoptosis. These results suggested that EZH2 may participate in tumor progression and influence cell function in LA cell lines. Moreover, we found that EZH2 was significantly expressed in the LA cell line resistant to cisplatin. EZH2 deletion sensitized cancer cells to cisplatin, leading to a significant decrease in survival of A549/DDP cells.

PcG genes (PcGs) play a key role in maintenance of cell identity. EZH2 gene, encoding a PcG protein, is also important in regulating cancer cell behaviour. The EZH2 protein is a member of the polycomb-complexes with histone lysine methyltransferase (HKMT) activity associated with transcriptional repression (15). Recent studies showed EZH2 was overexpressed in several human malignancies, and the level of EZH2 expression was correlated with cancer aggressiveness (7,16,17). The correlation between EZH2 expression

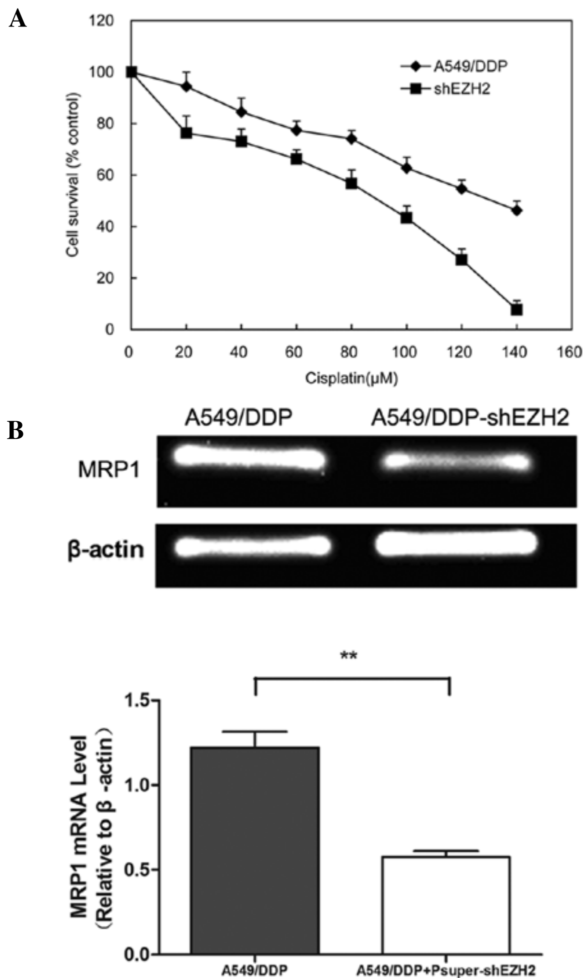

Figure 6. The effect of EZH2 depletion on DDP chemoresistance and mRNA expression of MRP1 in A549/DDP cells were analyzed. (A) The effect of EZH2 depletion on DDP chemoresistance. Cells were treated with various concentrations of DDP for 3 h followed by a 3-day recovery period. MTT assays were performed to assess chemoresistance. Cell survival was significantly inhibited after DDP treatment in A549/DDP-shEZH2 cells compared with control cells. The DDP IC_{50} was significantly lower in A549/DDP-shEZH2 (90.400 μM) cells vs. control cells (131.2 μM). (B) The effect of EZH2 depletion on mRNA level of MRP1. The relative level of MRP1 mRNA was downregulated in A549/DDP-shEZH2 cells compared with A549/DDP by RT-PCR. Data represent the mean and the standard deviation from three independent experiments. **P<0.01 vs. control cells.

and tumor aggressiveness/poor survival in those studies was consistent with our current observations in LA. Likewise, upregulated EZH2 expression was correlated with aggressive tumor behavior in patients with other malignant tumors such as prostate cancer (8), breast cancer (6) and gastric cancer (16). Our results extend the spectrum of tumors associated with EZH2. EZH2 expression is correlated with LA progression and may provide a novel way of diagnosis and prognosis of LA.

PcG proteins Bmi1 and EZH2 are epigenetic chromatin modifiers involved in cancer development (18), and the high EZH2 expression localized to primitive malignant cell types is often combined with a high Bmi1 expression (19,20). EZH2 may mediate increased invasiveness and metastasis by silencing downstream target adrenergic receptor β-2 (ADRB2) (21). Recent studies indicated that EZH2 mediated transcriptional silencing of a tumor suppressor gene E-cadherin by trimethylation of H3 lysine 27 (22,23). How the EZH2 gene affects downstream genes that are involved in oncogenesis and progression need to be clarified.

Recently, Hussain *et al* reported that tobacco smoke engaged polycomb repressor complexes that contained EZH2 to mediate epigenetic silencing of Dickkopf-1 (a Wnt signaling antagonist) and enhanced the malignant phenotype of LC cells (24), which was in good agreement with our current observations that high expression of EZH2 was related with smoking.

Cisplatin, a commonly used therapeutic agent in NSCLC, together with a third-generation anticancer drug, such as vinorelbine, gemcitabine or taxanes, is the standard regimen used in the first-line treatment of advanced NSCLC. However, LA is less chemosensitive than other NSCLC. Our findings were consistent with another study showing that loss of EZH2-mediated methylation of histone H3 lysine 27 (H3K27) re-sensitized ovarian cancer cells to cisplatin (25). It was demonstrated that overexpression of EZH2 contributed to acquired cisplatin resistance in ovarian cancer cells *in vitro* and *in vivo* (26). Our results identified EZH2 as an important factor in LA cell chemoresistance for the first time. Abbosh *et al* (25) found that H3K27 methylation played an important role in drug resistance. EZH2 deletion sensitized pancreatic cancer cells to doxorubicin and gemcitabine (27), because EZH2 mediated H3K27 methylation is a mark of heterochromatin, and loss of chromatin compaction could allow increased DNA damage at lower doxorubicin and gemcitabine doses leading to a decreased survival in EZH2 deleted pancreatic cancer cells. Based on the above, we will focus on a possible change in H3K27me3 levels with cisplatin resistance and how much it is reversed with EZH2 modulation in LA cells in our future studies.

We found that EZH2 downregulated the expression of MRP1 in LA cells. EZH2 deletion sensitized cancer cells to cisplatin, leading to a decrease of IC_{50}. Our results suggested that overexpression of EZH2 might play a role in LA chemoresistance. The combination usage of EZH2 inhibitors could be superior to conventional therapy, and an approach to the enhancement of chemoresistance in LA.

In summary, our experiments indicated that EZH2 expression was upregulated in human LA tissues and functionally linked to the malignant behavior of LA cells. These results suggested that high EZH2 expression may be one of the candidates for new molecular diagnosis, prognosis and therapeutic targets in LA. Our study provided novel information on the mechanisms underlying the progression and chemoresistance of LA, and indicates a demand for development of pharmacological inhibitor of EZH2 as a potential anticancer agent for chemotherapy of LA.

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