Effect of siRNA targeting *EZH2* on cell viability and apoptosis of bladder cancer T24 cells

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ABSTRACT. We investigated the effect of siRNA targeting enhancer of EZH2 on cell proliferation, invasion, migration, and apoptosis of human bladder cancer T24 cells. An siRNA-expressing plasmid targeting the *EZH2* gene was transfected into T24 cells. Quantitative polymerase chain reaction and Western blot analysis were used to detect EZH2 expression at the mRNA and protein levels, respectively. Proliferation, invasion, and migration of T24 cells were examined *in vivo* using MTT, wound healing, and transwell chamber migration assays, respectively. Annexin V-fluorescein isothiocyanate/propidium iodide flow cytometric analysis was performed to determine cell apoptosis levels. Expression of EZH2 in T24 cells was suppressed at the mRNA and protein levels. Following transfection for 48 h, growth was inhibited by 37.9%, which was markedly lower than that in the negative control group (P < 0.05). Following a wound-healing assay for 24 h, transfected cell migration distance was 1.37 ± 0.12, which was

markedly less than the horizontal migration distance of negative control group cells (P < 0.01). In addition, the cell invasion ability of EZH2-siRNA group cells decreased by 67% compared with negative control group cells (P < 0.01). Following transfection for 48 h, early- and late-stage apoptosis rates for T24 cells were 22.8 and 3.60%, respectively, which were higher than in the negative control group (P < 0.01). *EZH2* gene silencing effectively suppressed the proliferation, invasion, and migration abilities of human bladder cancer cells, promoting apoptosis.

Key words: Apoptosis; Bladder cancer; Enhancer of zeste homolog 2; biological behavior; siRNA

INTRODUCTION

Bladder cancer is one of the most common malignant tumors of the urinary system, and has the highest incidence and mortality rates among urological tumors in China. Recently, there has been a sharp increase in the incidence of bladder cancer. This disease affects more males than females at a ratio of 4:1. High rates of invasion, migration, and recurrence following surgery are the main biological characteristics of bladder cancer (Ploeg et al., 2009). Currently, the treatment of bladder cancer mainly involves surgery, radiotherapy, and chemotherapy; however, the overall curative effect is limited. Invasion and migration are the main causes of failure of bladder cancer treatment. Gene target therapy has attracted increasing attention in the clinic. Therefore, it is important to identify molecular targets for treating bladder cancer.

Enhancer of zeste homolog 2 (EZH2) from *Drosophila melanogaster* is an important member of the polycomb group, functioning as a specific methyltransferase of lysine 27 on histone H3. Bladder cancer is caused by the inhibition of genes associated with the negative regulation of proliferation (Chang and Hung, 2012). A number of studies have reported that excessive expression of EZH2 is associated with the susceptibility, development, and prognosis of various tumors, including non-small cell lung cancer (Zhang et al., 2013), colon cancer (Ferraro et al., 2013), acute lymphoblastic leukemia (Ntziachristos et al., 2012), prostate cancer (Shin and Kim, 2012), and ovarian cancer (Garipov et al., 2013). Recent studies have found that EZH2 is closely associated with the susceptibility and development of bladder cancer; however, the mechanisms associated with these functions remain unclear.

In the present study, small interfering RNA (siRNA) expression vectors were designed and constructed to target the *EZH2* gene. T24 bladder cancer cells were transfected, and the effect of the *EZH2* gene silencing on the proliferation, invasion, and migration capabilities and the apoptosis of bladder cancer cells was determined. The goal of this study was to establish a theoretical basis for identifying a new target for genetic therapy of bladder cancer.

MATERIAL AND METHODS

Materials and reagents

Bladder cancer T24 cells were obtained from the Key Laboratory of Kunming Medical University. RPMI-1640 culture medium, fetal bovine serum, 0.25% trypsin-EDTA, Dulbecco's phosphate-buffered saline, Lipofectamine 2000 transfection reagent, and enhanced

chemiluminescence detection reagent were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA), while dimethyl sulfoxide was purchased from Amresco, LLC (Solon, OH, USA). EZH2 antibody was purchased from Abcam (Cambridge, UK), and EZH2 siRNA was purchased from Ruibo Biotech Corporation (Guangzhou, China). Polyvinylidene fluoride membranes were purchased from Millipore (Billerica, MA, USA), while horseradish peroxidase-labeled goat anti-rabbit IgG secondary antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell culture

T24 bladder cancer cells were cultured in RPMI-1640 culture medium containing 10% fetal bovine serum in culture flasks with 5% CO_2 saturated humidity at 37°C. At 80-90% confluence, cells were harvested using 0.25% trypsin-0.53 mM ethylenediaminetetraacetic acid, inoculated onto 6-well plates, and cultured for a further 72 h. Cells were passaged using 0.1% trypsin.

Recombinant plasmid construction and transfection

Online design software was used to synthesize the following *EZH2*-specific siRNA sequence obtained from GenBank by gene sequence alignment: 5'-GGGAAAGUGUAUGAUA AUTT-3'. A negative reference siRNA sequence, 5'-UUCUCCGAACGUGUCACGUTT-3', was also designed. The synthesized sequences were inserted into the PGenesil -1.1 plasmid (Tiangen Biotech Co., Ltd., Beijing, China) to construct a recombinant plasmid, which was transformed into competent *Escherichia coli* DH5α cells (Tiangen Biotech). Individual colonies were selected and inoculated into RPMI-1640 culture medium containing 4 mg/mL neomycin. Following culture, plasmids were extracted by alkaline lysis, and enzyme digestion was performed for subsequent identification and sequencing analysis. Transfection was performed according to manufacturer instructions of the Lipofectamine 2000. Recombinant plasmids included a blank control, a negative plasmid, and an siRNA group.

RNA extraction following transfection and quantitative polymerase chain reaction (qPCR)

Three primers targeting *EZH2* siRNA were designed using the NCBI database (NM_004456 and NM_152998). Following transfection for 48 h, total RNA was extracted from T24 cells according to manufacturer instructions of the DP431 reagent (Tiangen Biotech). RNA integrity and concentration were determined and reverse transcription was performed to generate cDNA according to the cDNA reverse transcription kit (Invitrogen) instructions. cDNA from each group was used as a template to perform qPCR analysis of *EZH2* and β-*actin* gene expression levels. Reactions were repeated 3 times. At the same time, a template-free control was designed and analyzed by qPCR under the following reaction conditions: 15 s at 95°C, 5 s at 95°C, and 60 s at 60°C, for 30 cycles. Melting curve analysis was performed following amplification for 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C for 30 cycles. The specificity of the amplified product was analyzed by agarose gel electrophoresis. The $2^{-\Delta\Delta Ct}$ method was used for quantitative analysis. The following primers were used for analysis: for-

ward, 5'-GGTCTCCTCTGACTTCAACA-3' and reverse, 5'-GAGGGTCTCTCTCTTCCT-3' for β-*actin*; and forward, 5'-GCGCGGGACGAAGAATAATCAT-3' and reverse, 5'-TACACG CTTCCGCCAACAAACT-3' for *EZH2*.

Western blot analysis

RIPA protein lysate was used to extract protein from T24 cells 72 h after transfection, and proteins were then separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Next, proteins were transferred to polyvinylidene fluoride membranes and incubated with an anti-rabbit primary antibody against human EZH2 (1:100). Horseradish peroxidase-labeled goat anti-rabbit IgG secondary antibody (1:5000) was added and specific protein signal bands were revealed using an enhanced chemiluminescence detection reagent. Images were captured using Kodak X-OMAT BT film.

MTT cell proliferation assay

Cells obtained from passage 4-6 were grown to 90% confluence, and 0.25% trypsin -0.53 mM EDTA digestion solution was used to digest and count cells. Cells were inoculated onto a 96-well plate at a density of 2×10^3 cells/well and incubated at 5% CO_2 and 37°C for 18 h. Next, the culture medium was replaced and cells were incubated for a further 2 h prior to transfection with the *EZH2*-siRNA. After 24 h, cells were detected for 3 wells in each group; cells were then incubated at 5% CO_2 and 37°C for 30 min. Cells were then centrifuged, the supernatant was discarded, and 150 μL dimethyl sulfoxide was added. Following sufficient dissolution, absorbance at 490 nm was determined in each well using a microplate reader absorbance test plate. Absorbance was measured in 3 wells per group every 12 for 72 h and an average for each time point was determined to construct a cell growth curve.

Wound-healing assay

A wound-healing assay was performed to determine the migration abilities of the cells. A marker pen and ruler were used to draw horizontal lines across the wells (at least 5 lines per well) on the rear side of a 6-well plate at intervals of 0.5-1 cm. Each experimental group included 3 parallel samples. T24 cells were inoculated onto the 6-well plate at a density of 3×10^6 cells/well, and incubated at 5% CO_2 and 37°C for 24 h. Next, cells were transfected with *EZH2*-siRNA and cultured further. After 24 h, the medium was replaced with serum-free culture medium. At 100% confluence, a vertical scratch was made using a pipette tip aligned with a ruler to ensure that the tip remained vertical at all times. Phosphate-buffered saline was used to wash the cells 3 times to remove the scratched cells, serum-free culture medium was added, and the cells were incubated in 5% CO_2 at 37°C for 24 h. The scratch wound healing process was observed under an optical microscope (Olympus, Tokyo, Japan) and images were captured every 12 h.

Transwell assay

A transwell assay was used to detect the invasion abilities of the cells. Cells were

plated onto 6-well plates at a density of 5 x 10^5 cells/well and cultured for 24 h. Next, Lipofectamine 2000 was used to transfect the siRNA (final concentration, 100 nM) into the cells, and cells were incubated at 5% CO_2 and 37°C for 48 h. A negative control transfection was performed in 2 parallel wells. Following culture for 48 h, approximately 1 x 10^6 adherent cells were collected by digestion using 0.25% pancreatin (Tiangen Biotech) and prepared into a single-cell suspension. Matrigel (Invitrogen) was diluted to a concentration of 10 μg/250 μL (1:300) using serum-free MEM culture medium (Invitrogen) to produce an artificial substrate membrane adhesive for further use. Matrigel (3/80 μg per well) was added to a 24-well plate and dried overnight. Serum-free MEM cell culture solution was added to each well and incubated for 60-90 min to remove excess adhesive. EZH2 and negative-control transfected cells (2 x 10^5/well) were suspended in the upper chambers and incubated at 5% CO_2 and 37°C for 48 h. The culture solution was discarded from the transwell and washed with phosphate-buffered saline 3 times to remove non-adhered cells. The number of invaded cells on the rear side of the membrane was counted under an optical microscope following conventional fixation and colorization; 5 random fields were counted per well. An average was determined from 3 replicates per group.

Flow cytometry

Apoptosis levels were determined by flow cytometry. Trypsin digestion without EDTA was performed to collect T24 cells cultured and transfected for 24 h. Next, cells were centrifuged and washed, followed by addition of 500 μL binding buffer to the cell suspension. Annexin V-fluorescein isothiocyanate (5 μL), followed by 5 μL propidium iodide, were added to the cell suspension and mixed thoroughly. Apoptosis was detected by flow cytometry following 5-15 min incubation at room temperature in the dark.

Statistical analysis

The SPSS 10.0 software (SPSS, Inc., Chicago, IL, USA) was used to perform all statistical analyses. Data are reported as means ± standard deviation and were subjected to variance analysis. Groups were compared using the Student-Newman-Keuls method (q-test). $P < 0.05$ was considered to be statistically significant.

RESULTS

Effect of *EZH2*-siRNA on *EZH2* mRNA expression

T24 cells transfected with an siRNA-expressing plasmid targeting the *EZH2* gene were constructed as an experimental model to analyze the function of EZH2. To determine whether the transfected siRNA blocked *EZH2* expression, *EZH2* mRNA expression was determined following drug selection by qPCR. The PCR results showed that the *EZH2* expression in the 3 siRNA groups of cells was lower than that in the control groups, and this difference was statistically significant ($P < 0.05$). The strongest effect was observed in the *EZH2*-siRNA2 group, which exhibited the lowest expression of *EZH2*. *EZH2*-siRNA2 was used for subsequent experiments (Figure 1).
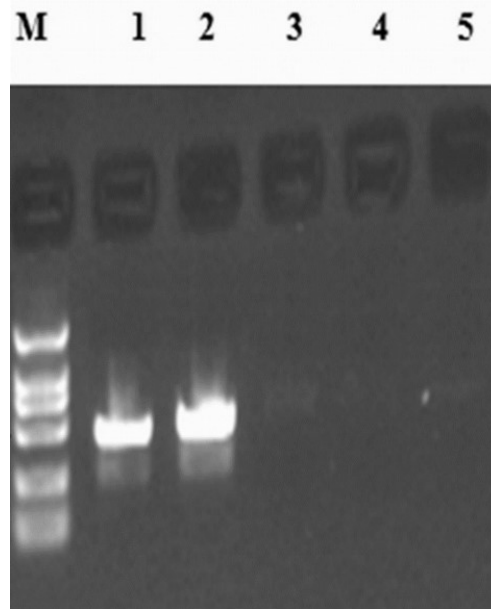
Figure 1. Expression of *EZH2* mRNA in T24 cells detected by qPCR. *Lane 1* = blank control; *lane 2* = negative control; *lane 3* = EZH2-siRNA1; *lane 4* = EZH2-siRNA2; *lane 5* = EZH2-siRNA3; *lane M* = marker. EZH2 = enhancer of zeste homolog 2.

Effect of *EZH2*-siRNA on EZH2 protein expression

Following down-regulation of EZH2 expression through siRNA in the T24 bladder cancer cells, EZH2 expression levels were silenced. The difference between EZH2 protein expression in the EZH2 siRNA and negative control siRNA groups was statistically significant (Figure 2).
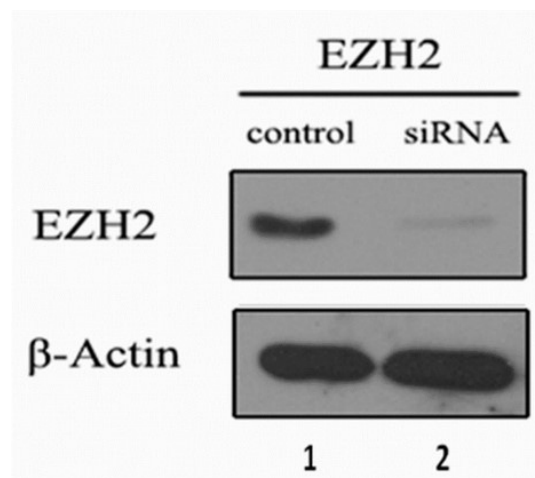

Figure 2. Expression of the EZH2 protein in T24 cells detected by Western blot analysis. *Lane 1* = negative control; *lane 2* = EZH2-siRNA. EZH2 = enhancer of zeste homolog 2; siRNA = small interfering RNA.

Effect of EZH2 expression silencing on the proliferation activity of T24 cells

The MTT assay results revealed that cell proliferation rate in the cells in the EZH2-siRNA group was reduced 24 h after transfection compared with that of the negative control group. The inhibition rates were 36.2, 37.1, 37.9, and 30.8%; compared to the negative control group, the differences were statistically significant ($P < 0.01$; Figure 3).
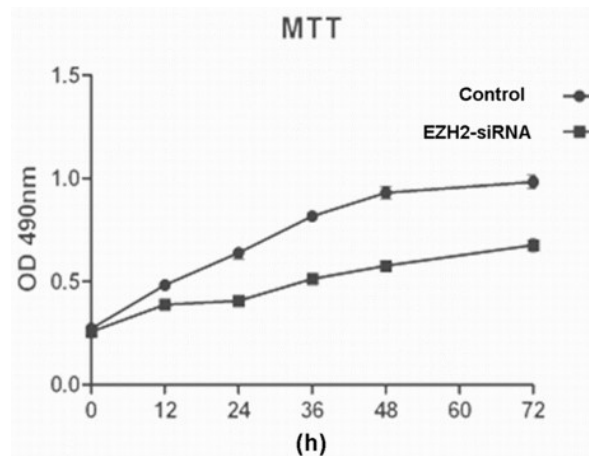

Figure 3. MTT cell proliferation assay was performed in bladder cancer cells transfected with EZH2-siRNA or negative control and a growth curve was constructed.

Migration capability of the cells following silencing of EZH2 expression

A wound healing assay revealed that 24 h after scratch treatment, the migration distances of the control and EZH2-siRNA-transfected cells were 1.98 ± 0.07 and 1.37 ± 0.12 cm, respectively. Compared with the negative control group, the horizontal migration distance of the EZH2-siRNA-transfected cells was significantly decreased ($P < 0.01$; Figure 4).

Effect of *EZH2* expression silencing on invasion capability of T24 cells

The transwell assay revealed that the number of cells invading the rear side of the membrane in the EZH2-siRNA group was markedly decreased compared with the control group. The invasion capability of the cells of the EZH2-siRNA group was significantly reduced by 67% compared with that of the negative control group ($P < 0.01$; Figure 5).

Effect of *EZH2* expression silencing on T24 cell apoptosis

Apoptosis levels were determined by flow cytometry. Annexin V/propidium iodide double staining revealed that the early and late apoptosis rates of the T24 cells in the negative control group were 7.31 and 2.49%, respectively, following 48 h of transfection. In contrast, the early and late apoptosis rates of cells in the EZH2-siRNA group were 22.8 and 3.60%, respectively. The difference between the respective apoptosis rates in the EZH2-siRNA and negative control groups was statistically significant ($P < 0.01$; Figure 6).
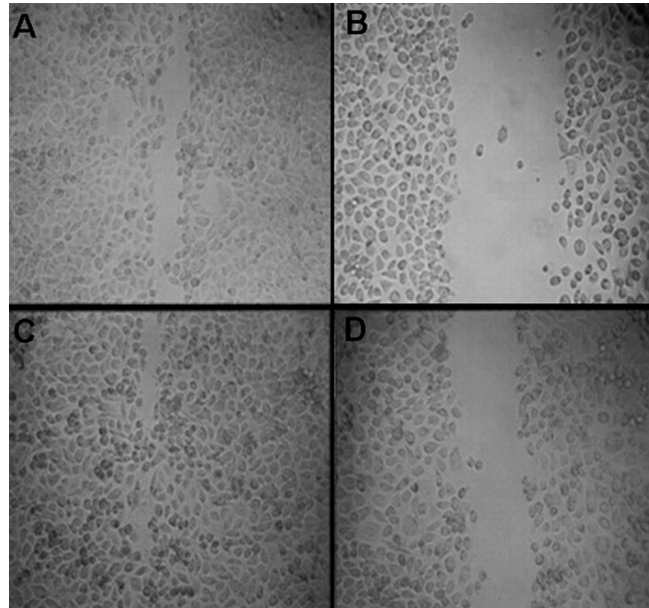
Figure 4. T24 cells were evaluated for their migratory phenotype in standard growth conditions by the scratch wound assay. **A.** Control group, 12 h. **B.** Transfection group, 12 h. **C.** Control group, 24 h. **D.** Transfection group, 24 h.
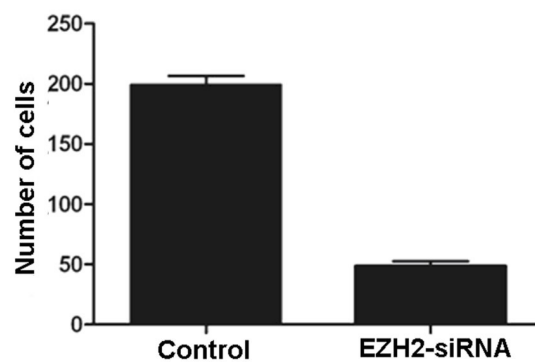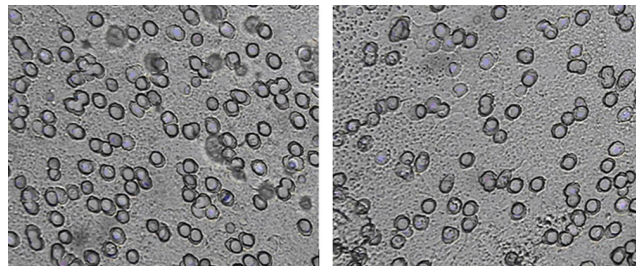

Figure 5. Effects of *EZH2* down-regulation on T24 cell invasion. EZH2, enhancer of zeste homolog 2; siRNA, small interfering RNA. **A.** Cells through the membrane. **B.** Number of cells through the membrane; left image was the control group and right image was the transfection group.
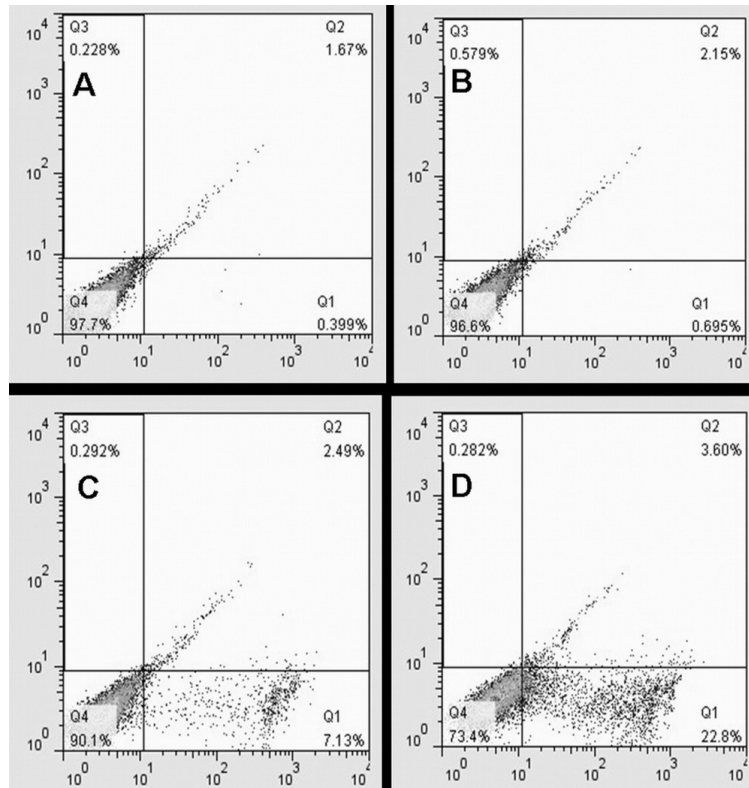
Figure 6. Effects of *EZH2* down-regulation on T24 cell apoptosis. EZH2 = enhancer of zeste homolog 2; siRNA = small interfering RNA. **A.** Control group, 0 h. **B.** Transfection group, 48 h. **C.** Control group, 0 h. **D.** Transfection group, 48 h.

DISCUSSION

The invasive capacity of malignant tumors is an interconnected, multistep, continuous, and complex biological process, the occurrence of which is closely associated with cell proliferation, differentiation, and apoptosis. Bladder cancer is the most common malignant tumor of the urinary system in China and has received significant clinical attention because of postoperative recurrence and migration. Therefore, understanding the molecular changes in bladder cancer cell invasion and migration is extremely important for the diagnosis, treatment, determination of prognosis, and improvement of survival rates of patients. Polycomb repressor complex 2 (PRC2) is a specific methyltransferase of lysine 27 on histone H3 and is associated with tumor development through the silencing, cell differentiation, and proliferation of inhibited genes. EZH2 is a core subunit of PRC2 and is involved in its catalytic function (Sauvageau and Sauvageau, 2010; Xiao, 2011). A number of previous studies identified low or absent expression of EZH2 in normal tissues, while its expression was found to be abnormally increased in tumor tissues (Takawa et al., 2011). Wan et al. (2013) quantitatively analyzed the correlation between EZH2 expression and lung cancer and found that the positive unit value of EZH2 was gradually increased with lung cancer development. This indicates that EZH2 is

very important in the development and migration process of lung cancer. EZH2 expression has also been proposed to represent an index of malignant glioma following qPCR and immuno-histochemical analyses in glioma tissues (Orzan et al., 2011). Through immunohistochemistry, Wang et al. (2013b) identified that the positive rate of EZH2 expression in kidney cell cancer was 67.9%, while the positive rate in normal kidney tissues was 28.6%. EZH2 was also found to be closely associated with the development of kidney cancer; therefore, monitoring the expression of EZH2 is useful for early diagnosis and prognostic evaluation.

siRNA has gained increasing attention in the research field of functional genomics and has been widely applied for functional research of tumor-associated genes. Specifically, in transitional cell carcinoma of the bladder, microRNA-10 was previously found to alter chromatin structure by directly inhibiting EZH2 expression, thus weakening the proliferative capability of tumor cells (Friedman et al., 2009; Bo et al., 2011). Wang et al. (2012) found that EZH2 was commonly expressed at high levels in transitional cell carcinoma of the bladder and positively correlated with the level of malignancy, indicating that the *EZH2* gene is an important determinant of transitional cell carcinoma development in the bladder. Thus, EZH2 may represent an effective biomarker of bladder cancer and may become a potential treatment target of bladder cancer. We investigated the effect of the *EZH2* gene on the activity and apoptosis of T24 bladder cancer T24 cells by silencing the *EZH2* gene using RNA interference. To clarify the function of EZH2 expression in the biological activity of T24 cells, we constructed a plasmid expression vector of siRNA against the human *EZH2* gene *in vitro* and the vector was transfected into T24 cells. Following transfection, the silencing effect of the vector was determined by analyzing EZH2 mRNA and protein levels by qPCR and Western blot analysis, respectively. EZH2 mRNA and protein expression was lower in the 3 siRNA groups compared with that of the control groups, enabling the further use of the *in vitro* experimental cell model for EZH2 gene function analysis.

The continuous division and unlimited proliferation capabilities of tumor cells are important determinants of malignancy. In the present study, the proliferative capabilities of T24 cells following *EZH2* gene silencing were determined using an MTT assay. Inhibition rates 24 h after transfection were 36.2, 37.1, 37.9, and 30.8%. The proliferation rate of cells in the EZH2-siRNA group was markedly slower than that in the negative control group, indicating that *EZH2* silencing inhibits the proliferative activity of T24 cells. The invasive capacity of tumor cells is important for determining the prognosis and survival rate of patients. In the present study, the invasive capacity of T24 cells is shown in Figure 6 as analyzed using the transwell assay. The number of T24 cells invading the rear side of the counting membrane was determined under an optical microscope. The number of invading cells in the EZH2-siRNA group was markedly decreased compared with that in the control group, and the invasion capability of cells in the EZH2-siRNA group was decreased by 67% compared with that in the negative control group. This indicates that the invasion capability of T24 cells transfected with EZH2-siRNA was markedly weakened.

Tumor cells require considerable athletic ability to separate from parent tumors, penetrate through blood vessel walls, and invade normal tissues. In this study, EZH2 was found to be closely associated with the migration of bladder cancer cells. Previously, Luo et al. (2013) identified that long-chain non-coding RNA-H19 inhibits E-cadherin expression by associating with EZH2, which increases migration and invasion. In contrast, following *EZH2* silencing, E-cadherin expression is induced, reducing the invasion and migration of tumor cells to improve the prognosis of patients and increase survival rates (Wang et al., 2013a). In the present

study, an *in vitro* cell injury healing model was used to observe the migratory characteristics of tumor cells on an extracellular matrix. The results revealed that, 24 h after scratch treatment, the transfer distances of the control and EZH2-siRNA group cells were 1.98 ± 0.07 and 1.37 ± 0.12, respectively. Compared with the negative control group, the horizontal migration distance of the cells of the EZH2-siRNA group was markedly decreased; the shortened trace distance was also decreased. These observations indicate that the invasive capability of the T24 cells transfected with EZH2-siRNA was markedly weakened.

　　　　To determine the effect of *EZH2* gene silencing on T24 cell apoptosis, Annexin V/ propidium iodide double staining was performed. The results showed that the early and late apoptosis rates in the EZH2-siRNA group were 22.8 and 3.60%, respectively, following 48 h of transfection. These rates were increased compared with those of the negative control group, particularly for early stage apoptosis, indicating that EHZ2 gene silencing increases T24 cell apoptosis. Thus, EZH2-siRNA may be used to directly treat bladder cancer T24 cells.

　　　　In conclusion, the *EZH2* gene is closely associated with the activity and apoptosis of the T24 bladder cancer cells. In the present study, targeted inhibition of EZH2 expression in the T24 bladder cancer cells was found to inhibit the proliferation, invasion, and migration capabilities of the bladder cancer cells, and to promote apoptosis. These results provide a theoretical basis for understanding the molecular regulation mechanisms of EZH2 during the invasion and migration of T24 bladder cancer cells. In addition, these observations highlight the potential of EZH2 as a biomarker of T24 bladder cancer cells and a new target for therapy.

ACKNOWLEDGMENTS

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