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EZH2 promotes gastric cancer cells proliferation by repressing p21 expression

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ABSTRACT

EZH2 is a core component of the polycomb repressive complex 2 (PRC2), which catalyzes trimethylation of histone H3 lysine 27 (H3K27me3) and promotes carcinogenesis by epigenetically silencing many tumor suppressor genes. Increased EZH2 expression is a marker of advanced and metastatic in many cancers, including lung, prostate and breast cancer, and it has been considered as a potential novel therapeutic target. However, the clinical significance and molecular mechanisms of EZH2 controlling gastric cancer cell proliferation and invasion are not well documented. In this study, immunohistochemical analysis was conducted to investigate the EZH2 expression in gastric cancer. We found that EZH2 levels were increased in gastric cancer tissues compared with adjacent normal tissues. Moreover, patients with high levels of EZH2 expression had a relatively poor prognosis. Furthermore, knockdown of EZH2 expression by siRNA could impair cell proliferation and invasion both in *vitro* and *vivo*. Finally, we found that EZH2 influences gastric cancer cells proliferation partly through regulating p21 expression. Our findings present that EZH2 over-expression can be identified as a poor prognostic biomarker in gastric cancer.

1. Introduction

Gastric cancer is one of the most common human cancers and still remains the second most common cause of cancer mortality worldwide, despite decreasing incidence and mortality rates in developed countries over the past 20 years [1,2]. Although the majority of the patients at an early stage of gastric carcinoma can be cured by surgery, more than half of those at an advanced stage of this disease die of carcinoma recurrence, even after undergoing curative gastrectomy [3]. In spite of a great advancement on the research of gastric cancer, the molecular mechanisms underlying gastric cancer development and metastasis are still poorly understood. Therefore, a better understanding of the pathogenesis and identification of the molecular alterations is essential for the development of diagnostic markers that aid novel effective therapies for gastric cancer [4,5].

Enhancer of Zeste Homolog 2 (EZH2) is a core component of the Polycomb Repressive Complex2 (PRC2) which also includes SUZ12 and EED. PRC2 represses gene transcription through trimethylation of Lys27 of histone H3 (H3K27), and contribute to the maintenance of cell identity, cell cycle regulation and oncogenesis [6,7]. Recently, increasing evidence reveals that EZH2 overexpression occurs in a variety of human malignancies including non small cell lung cancer [8], colorectal cancer [9], hepatocellular [10] and breast cancers [11]. More importantly, abnormalities of EZH2 expression were observed to correlate closely with tumor aggressiveness or poor patient prognosis. For example, EZH2-mediated inactivation of KLF2 blocks the tumor-suppressor features of the KLF2 and its growth-inhibitory features in both cellular and animal models [12]. Moreover, EZH2 supports nasopharyngeal carcinoma cell aggressiveness by inhibit E-cadherin transcription through forming a co-repressor complex with HDAC1/HDAC2 and Snail [13].

To date, the potential oncogenic roles and molecular mechanisms of EZH2 in gastric cancer has not been clearly documented. In this study, we found that EZH2 levels were significantly over-expressed in gastric cancer tissues, and its expression level was correlated with patients poor prognosis. Furthermore, knockdown of EZH2 impaired cells proliferation partly through regulating p21 expression. Our findings present that EZH2 over-expression can be identified as a poor prognostic

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biomarker in gastric cancer.

2. Materials and methods

2.1. Cell lines and culture conditions

Five gastric cancer cell lines(SGC7901, AGS, MGC803, MKN45, MKN28 and BGC823), and a normal gastric epithelium cell line (GES-1) were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). GES-1, SGC7901, MKN45 and MKN28 cells were cultured in DMEM (GIBCO-BRL) medium ; MGC803 and BGC823 cells were cultured in RPMI 1640 (GIBCO-BRL); AGS cells were cultured in F12 (GIBCO-BRL) medium supplemented with 10% fetal bovine serum (10% FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen) at 37 °C with 5% CO2.

2.2. Immunohistochemical analysis

To determine the expression level of EZH2 in gastric cancer tissues, paraffin-embedded formalin-fixed tissues were immunostained for EZH2. The immunohistochemical staining was performed on an automated staining system (Techmate 500, DakoCytomation) with a rabbit anti-EZH2 antibody (1:100,CST) for 30 min. For immunohistochemical measurement of EZH2 expression, the signal was amplified and visualized with diaminobenzidine-chromogen, followed by counterstaining with hematoxylin. For EZH2, an IHC score with 2+ or more was defined as positive, while IHC scores with 0 and 1+ were defined as negative. All the patients information was shown in Table S1.

2.3. RNA extraction and qPCR analyses

Total RNA of gastric cancer cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). For qPCR assay, 1 μg RNA was reverse transcribed into cDNA by using a Reverse Transcription Kit (Takara, Dalian, China). QPCR analyses were performed with Power SYBR Green (Takara, Dalian China), and the results were normalized to the expression of GAPDH. The PCR primers for P21 or GAPDH were following: GAPDH sense, 5′-GTCAACGGATTTGGTCTGTATT-3′ and reverse, 5′ -AGTCTTCTGGGTGGCAGTGAT-3′; P21 sense, 5′-AGCTGCCGAAGTCAGTTCCTT-3′, reverse, 5′-GTTCTGACATGGCGC CTCCT-3. QPCR and data collection were performed on ABI 7500.The relative expression of EZH2 was calculated and normalized using the $2^{-\Delta\Delta Ct}$ method relative to GAPDH.

2.4. Plasmid constructs

To generate a EZH2 shRNA vector, We also designed shRNA sequence targeted human EZH2. After annealing of the complementary shRNA oligonucleotides, we ligated the annealed oligonucleotides into plko vector (sh-EZH2)(Sigma, St. Louis, Mo).

2.5. Transfection of gastric cancer cells

All plasmid vectors (sh-EZH2 and empty vector) for transfection were extracted using DNA Midiprep kit (Qiagen, Hilden, Germany). Cells cultured on six-well plate were transfected with the sh-EZH2, empty vector, si-EZH2 or si-NC using Lipofectamine2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. 48 h post transfection, the cells were harvested for qPCR and western blot analyses. siRNA for the human EZH2 (2# 5′-AUCAGCUCGUCUGAACCU CUU-3′, 3#5′-GAGGUUCAGACGAGCUGAU-3′), shRNA for EZH2 (GGGCCAGACTGGGAAGAAA) and the negative control siRNA (5′-UUC UCCGAACGUGUCACGUUU-3′) were purchased from Invitrogen.

2.6. Cell proliferation assays

A cell proliferation assay was performed with MTT kit (Sigma, St. Louis, Mo) according to the manufacturer's instruction. Cells were placed into 6-well plate and maintained in medium containing 10% FBS for 2 weeks. Colonies were fixed with methanol and stained with 0.1% crystal violet (Sigma, St. Louis, Mo). Visible colonies were manually counted.

2.7. Cell migration and invasion assays

For the transwell assays, 5×10^4 (migration) or 1×10^5 (invasion) cells in serum-free media were placed into the upper chamber of an insert (8-μm pore size; Millipore, Billerica, MA, USA). Medium containing 10% FBS was added to the lower chamber. After incubation for 24 h, the cells remaining on the upper membrane were removed with cotton wool. Cells migrated or invaded through the membrane were stained with 0.1% crystal violet, imaged and counted using an IX71 inverted microscope (Olympus, Tokyo, Japan).

2.8. Tumor formation assay in a nude mouse model

4 weeks female athymic BALB/c nude mice were maintained under pathogen-free conditions and manipulated according to protocols approved by the Shanghai Medical Experimental Animal Care Commission. SCG7901 cells transfected with sh-EZH2 and empty vector were harvested and washed with PBS, and resuspended at a concentration of 1×10^8 cells/mL. A volume of 100 μL of suspended cells was subcutaneously injected into a single side of the posterior flank of each mouse. Tumor growth was examined every three days, and tumor volumes were calculated using the equation $V = 0.5 \times D \times d2$ (V, volume; D, longitudinal diameter; d, latitudinal diameter). At 18 days post injection, mice were euthanized and the subcutaneous growth of each tumor was examined. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Huzhou Central Hospital.

2.9. Western blotting analysis

Cells were lysed with RIPA protein extraction reagent (Beyotime, Beijing, China) supplemented with a protease inhibitor cocktail (Roche, CA, USA). The concentration of protein was determined using the Bio-Rad protein assay kit. Protein extracts (40 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to nitrocellulose membranes (Sigma) and incubated with specific antibodies. Autoradiograms were quantified by densitometry using Quantity One software (Bio-Rad, CA, USA). Antibodies against P21 (1:1000 dilution) and GAPDH were purchased from Cell signaling.

2.10. Statistical analysis

Statistical analysis was performed using the SPSS software package (version 20.0, SPSS Inc). Statistical significance was tested by a Student's *t*-test or a Chi-square test as appropriate. Survival analysis was performed using the Kaplan-Meier method, and the log-rank test was used to compare the differences between patient groups.

3. Results

3.1. EZH2 is over-expressed and correlated with poor prognosis in human gastric cancer tissues

We firstly examined EZH2 expression in 89 paired gastric cancer

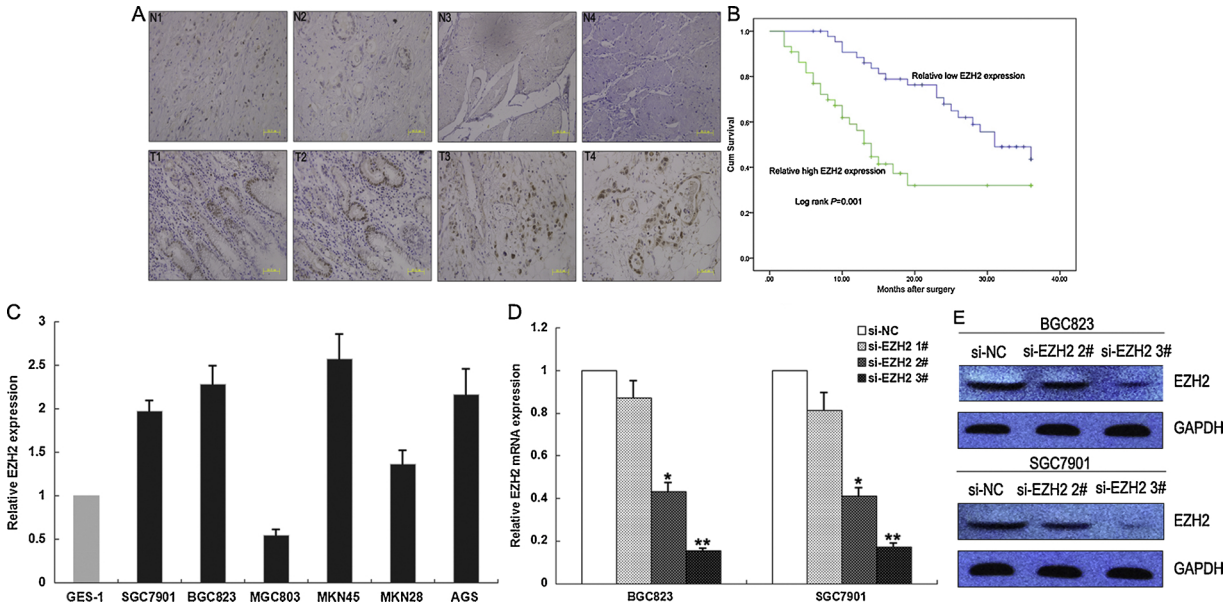

Fig. 1. Relative EZH2 expression in gastric cancer tissues and its clinical significance. a) Relative expression of EZH2 protein in gastric cancer tissues (n = 89) in comparison with corresponding non-tumor normal tissues (n = 89). EZH2 protein expression was examined by Immunohistochemistory. b) Kaplan–Meier overall survival curves according to EZH2 expression level. c) EZH2 levels in gastric cancer cell lines (SGC7901, AGS, MKN-45, MKN-28, MGC803 and BGC823) and normal human gastric epithelial cell line (GES-1) were detected by qPCR. d) QPCR and western blot analyses of EZH2 expression level following treatment BGC823 and SGC7901 cells with si-EZH2. *P < 0.05, **P < 0.01.

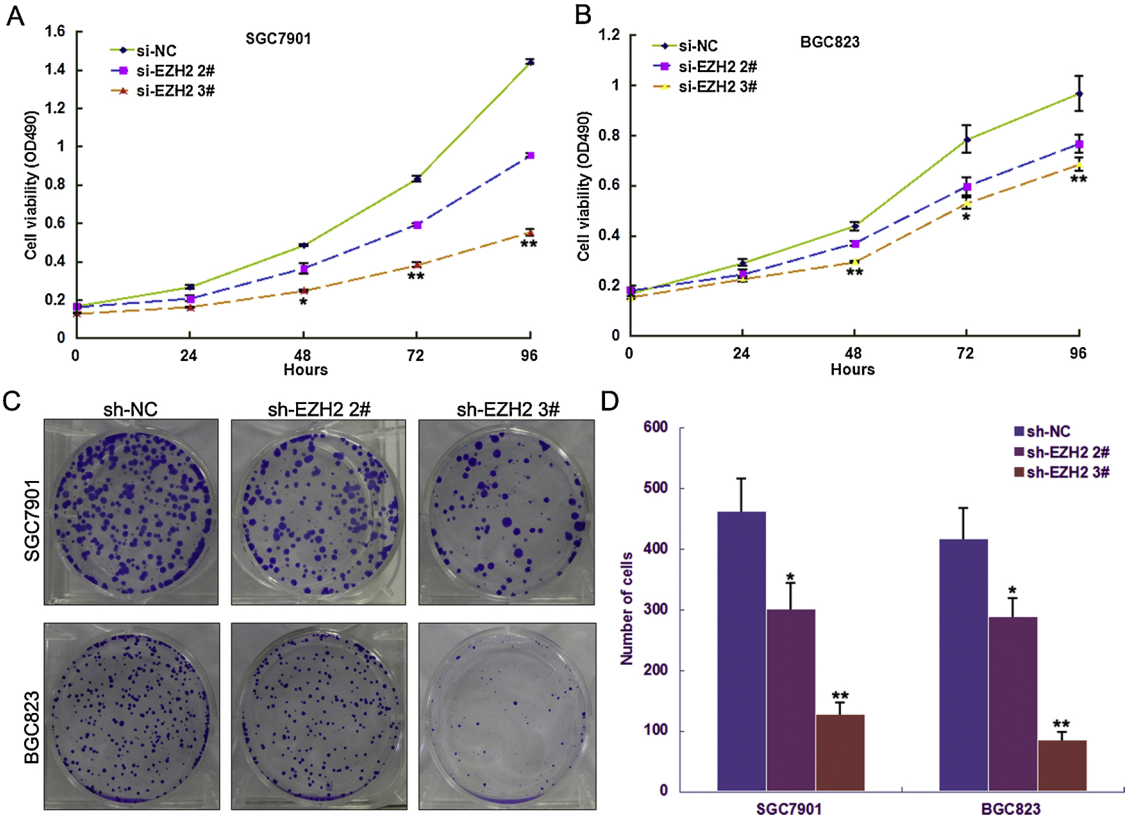

Fig. 2. The effect of EZH2 down-regulation on gastric cancer cells proliferation *in vitro*. a,b) MTT assay was performed to determine the proliferation of si-EZH2 transfected BGC823 and SGC7901 cells. Data represents the mean ± s.d. from three independent experiments. c,d) Colony-forming growth assay was performed to determine the colony formation ability of sh- EZH2 transfected BGC823 and SGC7901 cells. The colonies were counted and captured. *P < 0.05 and **P < 0.01.

samples and adjacent, histological normal tissues by Immunohistochemistory. The results showed that EZH2 was significantly up-regulated in gastric cancer tissues compared with corresponding adjacent non-tumorous tissues (Fig. 1A). According to the median ratio of relative EZH2 expression in tumor tissues, the 89 gastric cancer patients were classified into two groups: relative High-EZH2 group (n = 45, EZH2 expression ratio ≥ median ratio) and relative Low-EZH2 group (n = 44, EZH2 expression ratio < median ratio).

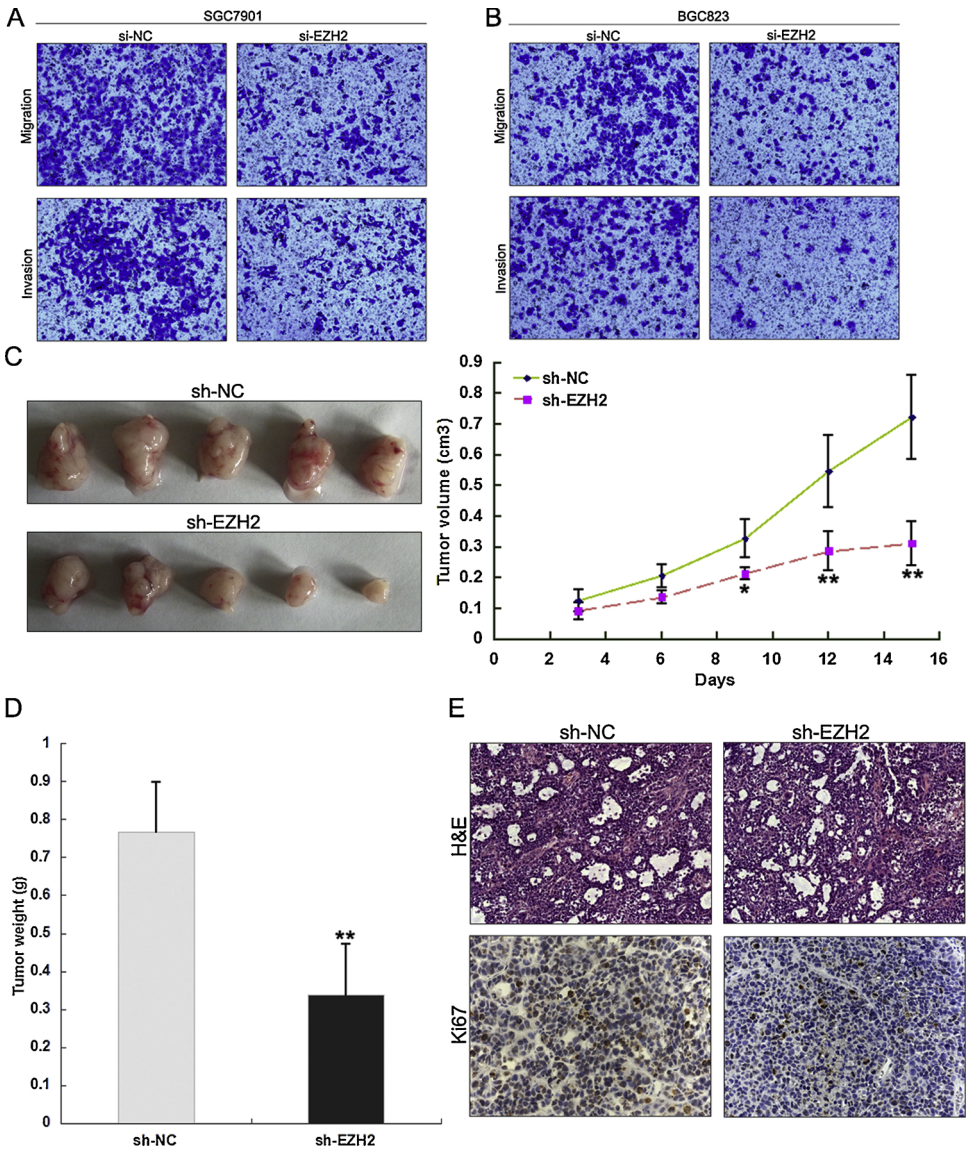

Fig. 3. The effect of inhibition of EZH2 on gastric cancer cells migration, invasion and tumor growth *in vivo*. a,b) Transwell assays were used to investigate the changes in migratory and invasive abilities of BGC823 and SGC7901 cells. c) The tumor volume was calculated every three days after injection of SGC7901 cells stably transfected with sh-EZH2 or empty vector. d) Tumor weights are represented as means of tumor weights ± s.d. **e** Tumors developed from sh-EZH2 transfected SGC7901 cells showed lower ki67 protein levels than tumors developed by control cells. Upper: H & E staining; Lower: immunostaining. *P < 0.05, **P < 0.01 and N.S. not significant.

Kaplan-Meier survival analysis and log-rank tests using patient post-operative survival were performed to further investigate the correlation between EZH2 expression and gastric cancer patient prognosis. With regard to overall survival, patients with higher EZH2 expression had a significantly poorer prognosis than those with lower EZH2 expression (P = 0.001, log-rank test) (Fig. 1B).

3.2. Manipulation of EZH2 expression level in gastric cancer cells

To evaluate the biological functions of EZH2, we first examined the expression of EZH2 in a variety of cell lines, including SGC7901, BGC823, MGC803, MKN45, MKN28, AGS and normal gastric epithelium cell line GES-1 by qPCR. The results showed that EZH2 expression was significantly up-regulated in gastric cancer cells (Fig.1C), suggesting that a increase in expression levels may be significant in oncogenesis. In order to manipulate EZH2 level in gastric cancer cells, EZH2 siRNAs were transfected into SGC7901 and BGC823 cells to down-regulate endogenous EZH2 expression. qPCR and western blot analyses revealed that EZH2 expression was decreased in si-EZH2 2# and 3# transfected cells when compared with control cells (Fig.1D).

3.3. Knockdown of EZH2 inhibits gastric cancer cells proliferation in vitro

To assess the biological role of EZH2 in gastric cancer, we investigated the effect of targeted knockdown of EZH2 on cell proliferation. MTT assays revealed that cell growth was significantly impaired in si-EZH2 transfected SGC7901 cells and BGC823 cells (Fig. 2A and B). Similarly, the results of colony-formation assays revealed that clonogenic survival was decreased following down-regulation of EZH2 in sh-EZH2 transfected SGC7901 cells and BGC823 cells (Fig.2C and D).

3.4. Down-regulation of EZH2 inhibits migration and invasion of gastric cancer cells

Furthermore, we evaluated cancer cell invasion and migration ability through transwells assay. Decreased EZH2 expression levels impeded the migration of SGC7901 and BGC823 cells compared with controls. Similarly, invasive ability of SGC7901 and BGC823 cells was also reduced following down-regulation of EZH2 expression (Fig. 3A and B).

3.5. Knockdown of EZH2 inhibits gastric cancer cells tumorigenesis in vivo

To explore whether the level of EZH2 expression could affect

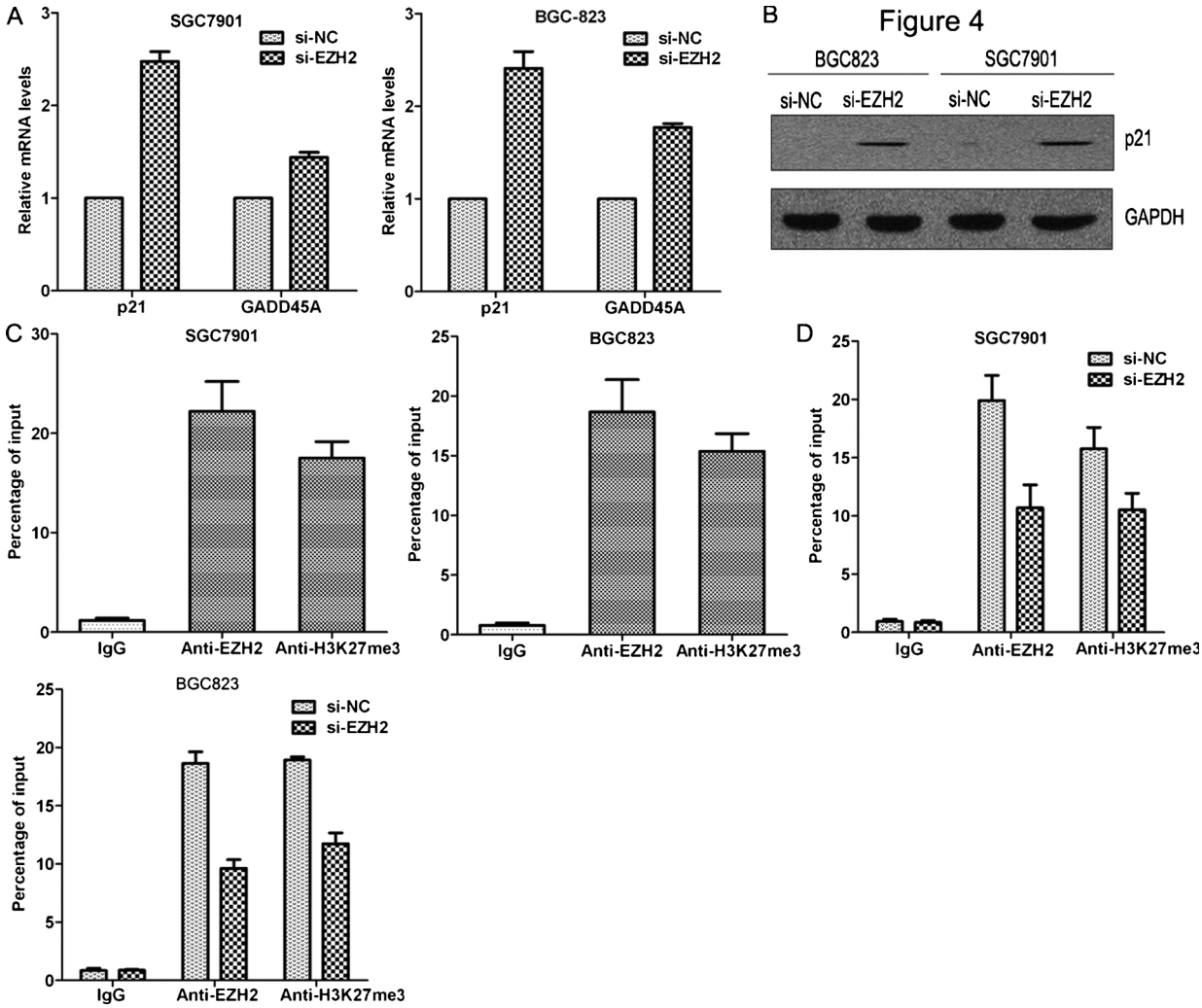

Fig. 4. EZH2 repressed P21 expression by directly binding to its promoter regions. a) QPCR assays were performed to detect P21 and GADD45 A expression in BGC823 and SGC7901 cells transfected with si-EZH2. b) Western blot analysis of P21 protein levels in BGC823 cells transfected with si-EZH2. c) ChIP–qPCR of EZH2 and H3K27-3me occupancy binding in P21 promoter in BGC823 and SGC7901cells. d) ChIP–qPCR of EZH2 and H3K27-3me binding in the P21 promoter in BGC823 and SGC7901 cells treated with EZH2 siRNA (48 h) or scrambled siRNA. *P < 0.05, **P < 0.01.

tumorigenesis, sh-EZH2 or empty vector stably-transfected SGC7901 cells were inoculated into nude mice. Eighteen days after injection, the tumors formed in sh-EZH2 group were substantially smaller than those in the control group (Fig. 3C). Moreover, the mean tumor weight at the end of the experiment was markedly lower in the sh-EZH2 group compared to the empty vector group (Fig. 3D). Immunostaining showed that Ki67 levels in tumors formed from sh-EZH2 transfected SGC7901 cells were lower than in tumors from control cells (Fig. 3E).

3.6. P21 is key downstream mediators of EZH2

Further exploration of the underlying mechanisms involved in EZH2 knockdown induced growth arrest was done by examining the expression of P21 and GADD45 A after transfection with si-EZH2 or si-NC. The results showed that P21 was significantly increased in si-EZH2 transfected cells compared to control cells (Fig. 4A). Moreover, increased P21 protein level were observed in cells transfected with si-EZH2 compared to control cells (Fig. 4B). Furthermore, the results of ChIP assays revealed that EZH2 could directly bind to P21 promoter region and mediate H3K27me3 modification, while knockdown of EZH2 decreased the binding and modification (Fig. 4D). These data confirm that EZH2 functions as an oncogene by regulating P21 in gastric cancer cells.

4. Discussion

Generally, the development of cancer arise from gene mutations or altered gene expression, which eventually cause dysregulation of numerous important oncogens, tumor suppressor proteins and noncoding RNAs [14]. Chancges in genes expression involved in not only genetic and environmental factors but also epigenetic factors that do not affect the primary DNA sequence [15,16]. Epigenetic alterations of chromatin include DNA methylation or demethylation as well as altered patterns of histone modifications, which can affect gene-expression profiles and contribute to the formation and progression of cancer [17–19]. As an important subunit of PRC2, EZH2 is over-expressed in multiple cancers and silences a lot of tumor suppressor genes transcription [20]. For example, over-expression of EZH2 induced H3K27me3 modification in Ovarian Cancer is associated with epigenetic repression of the ARHI tumor suppressor gene [21]. Moreover, EZH2 dependent H3K27me3 is also involved in epigenetic silencing of ID4 in prostate cancer [22]. These findings indicate that EZH2 plays an important role in cancer development and progression.

In this study, we found that EZH2 was significantly over-expressed in gastric cancer tissues and cells, and increased EZH2 expression was correlated with patients shorter overall survival. Moreover, knockdown of EZH2 inhibited gastric cancer cells proliferation and impaired cell

migration and invasion *in vitro* and *in vivo*. Meanwhile, a lot of previous studies showed that EZH2 can repress many important tumor suppressor genes expression and contribute to cancer cells proliferation and metastasis [23–25]. For example, EZH2 could promote the activation of wnt signaling in gastric carcinogenesis through down-regulation of CXXC4 expression [26]. In the present study, we found that EZH2 involved in gastric cancer cells proliferation partly via regulating P21 expression.

P21, a member of the Cip/Kip family of cyclin kinase inhibitors (CKIs), acts as a master effector of multiple tumour suppressor pathways for anti-proliferative activities [27]. In addition, P21 mediates biological activities primarily by binding to and inhibiting the kinase activity of the cyclin-dependent kinases (CDKs), which results in growth arrest at specific stages in the cell cycle [28]. Dysregulated p21 expression often correlates with the loss of p21 transcriptional activators (including p53) and up-regulation or mutations of p21 transcription suppressors. Therefore, the regulation of p21 transcription is complex. A variety of transcription factors, such as p53, activator protein 2, STATs and CCAAT/enhancer binding protein alpha, can regulate P21 transcription in response to different signals [29,30]. In the present study, we found that epigenetic alteration, such as H3K27me3 modification mediated by EZH2 also contributes to transcription repress of P21 in gastric cancer cells.

In summary, the expression of EZH2 was significantly increased in gastric cancer tissues, suggesting that its up-regulation may be a negative prognostic factor for gastric cancer patients. We showed that EZH2 possibly regulates the proliferation ability of gastric cancer cells, partially through regulation of P21. Our findings further the understanding of gastric cancer pathogenesis and development, which may facilitate the development of diagnostics and therapeutics against gastric cancer.

Conflicts of interest

The authors declare that they have no competing interests.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.prp.2019.03.003.

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