Article

J. Biol. Regul. Homeost. Agents. 2024; 38(10): 5927–5936
https://doi.org/10.23812/j.biol.regul.homeost.agents.20243810.472

Effect of Polycomb Repressive Complex 2 on the Proliferation, Migration, Invasion, and Apoptosis of Gastric Cancer Cells by Targeting H3K27 Activation

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Submitted: 31 October 2022 Revised: 8 October 2023 Accepted: 10 October 2023 Published: 1 October 2024

Background: Gastric cancer is a globally prevalent malignancy characterized by dysregulated cellular processes including epigenetic modifications. However, the Polycomb Repressive Complex 2 (PRC2), a pivotal epigenetic regulator, modulates gene expression through trimethylation of histone H3 at lysine 27 (H3K27me3), thereby orchestrating cellular identity and function. Therefore, this study aimed to elucidate the impact of PRC2 and H3K27me3 on critical cellular behaviors, such as proliferation, migration, invasion, and apoptosis, which holds the potential to unveil novel insights into gastric cancer progression.

Methods: Human gastric cancer cells NCI-N87 were seeded in a 6-well plate and were divided into the normal, siRNA-negative control (siRNA-NC), siRNA-Enhancer of zeste homolog 2 (siRNA-EZH2), and siRNA-SUZ12 polycomb repressive complex 2 subunit (siRNA-SUZ12) groups. The cells were transfected to knock down the expression of PRC2 core subunits, siRNA-Enhancer of zeste homolog 2 (EZH2) and SUZ12 polycomb repressive complex 2 subunit (SUZ12). Moreover, Western Blot analysis and Quantitative real-time reverse-transcription PCR (qRT-PCR) were carried out to determine the expression levels of the EZH2 and SUZ12 in gastric cancer cells. Furthermore, the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was employed to examine cell proliferation, the Transwell assay was employed to determine cell migration and invasion, and flow cytometry was used to evaluate cell apoptosis rate. Additionally, the confocal laser scanning technique was utilized to assess H3K27 methylation in gastric cancer cells. Finally, the interaction between PRC2 and H3K27 was evaluated using co-immunoprecipitation (Co-IP).

Results: Compared to the siRNA-NC group, there was a significant decrease in the levels of EZH2 protein and mRNA in the cells of the siRNA-EZH2 group and SUZ12 protein and mRNA in the siRNA-SUZ12 group ($p < 0.05$). Furthermore, both the siRNA-EZH2 and siRNA-SUZ12 groups showed significantly reduced cell survival rates, and decreased count of migrating and invading cells, while exhibited significantly increased apoptosis rate compared to the siRNA-NC group. Moreover, the expression level of H3K27me3 significantly elevated in these cells ($p < 0.05$). Additionally, Co-IP results revealed a significant interaction of EZH2 and SUZ12 with H3K27me3.

Conclusion: This study delved into the impact of the PRC2 on gastric cancer cell behavior, focusing on the targeted regulation of histone H3K27me3. The findings suggest that PRC2 might modulate cellular proliferation, migration, invasion, and apoptosis within gastric cancer cells. Nevertheless, rigorous experimental validation is essential to establish causal relationship and gain deeper mechanistic insights into PRC2's role in gastric cancer.

Keywords: gastric cancer; PRC2; H3K27; proliferation; migration; invasion; apoptosis

Introduction

Gastric cancer is a globally prevalent malignancy, posing a significant health risk to Chinese [1]. Currently, surgery combined with radiotherapy and chemotherapy is an approach for managing gastric cancer. However, most of the patients are considered unsuitable for surgery when diagnosed, and the available treatments, such as radiotherapy and chemotherapy are associated with toxic side effects and collateral damage to healthy tissues, resulting in limited efficacy [2].

With the development of the molecular basis of tumor genesis, molecular targeted therapy demonstrates great potential for anti-tumor treatment. Polycomb Repressive Complex 2 (PRC2), a member of the Polycomb Group (PcG), contains two core subunits, siRNA-Enhancer of zeste homolog 2 (EZH2) and SUZ12 polycomb repressive complex 2 subunit (SUZ12) [3]. Previous research has shown that EZH2 can trigger the trimethylation of histone histone H3 at lysine 27 (H3K27me3), activating downstream silenced genes to participate in tumorigenesis [4]. Additionally, it has been reported that jumonji, AT rich interactive domain 2 (JARID2) and Elongin BC and Poly-

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comb repressive complex 2-associated protein (EPOP), two unique structural platforms within SUZ12, determine the diverse types of chromatin binding PRC2. Disruption in JARID2-SUZ12 interaction might contribute oncogenic chromosome translocation involving SUZ12 [5]. Therefore, the development of small-molecule drugs targeting the function of PRC2 holds critical clinical importance for the treatment of malignant tumors, including gastric cancer [6]. In the current study, human gastric cancer NCI-N87 cells and siRNA technology were employed to assess the impact of PRC2 on the proliferation, migration, invasion, and apoptosis of gastric cancer cells. Confocal laser scanning was used to evaluate the level of H3K27 methylation modification by PRC2. Furthermore, the co-immunoprecipitation (Co-IP) technique was employed to analyze the interaction between PRC2 and H3K27 within cells.

Materials and Methods

Materials

Main Reagents and Cells

Human gastric cancer NCI-N87 cells (Item No. PX274), electrochemiluminescence (ECL) detection kit (Item No. PR2070), Annexin V-FITC/PI (Propidium Iodide) apoptosis kit (Item No. PR1704), Triton X-100 (No. PR1238), liquid 4′,6-diamidino-2-phenylindole (DAPI) (Item No. PR1745), and TRIzol agent (Item No. PC2170) were obtained from Beijing Pufei Biological. Roswell Park Memorial Institute-1640 (RPMI-1640) medium containing 10% of fetal bovine serum (FBS) (Item No. PM150110B) was purchased from Wuhan Proceeds Biology. Lipofectamine 2000 reagent (Item No. 11668) was obtained from Invitrogen, USA. siRNA-EZH2 and siRNA-SUZ12 were obtained from OriGene Technologies, USA. 1× PBS solution (Item No. DD547050), Tris-buffered saline with 0.1% Tween® 20 detergent (TBST) solution (Item No. DD547114), and 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) kit (Item No. YIJI10039) were purchased from Shanghai Yiji Industry. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) Protein Loading Buffer (Item No. ZN1876). The bicinchoninic acid (BCA) Protein Quantitation Kit (Item No. GL1484), Rabbit Anti-Human β-actin Monoclonal Antibody (Item No. SY1068), and Protein A/G Plus Agarose (Item No. GS4780) were provided by Beijing Biolabs. Rabbit anti-human EZH2 polyclonal antibody (Item No. ZA-0133) was purchased from Beijing NobleRyder. Rabbit anti-human SUZ12 polyclonal antibody (Item No. ab12073) and Rabbit anti-human H3K27me3 monoclonal antibody (Item No. ab176916) were provided by Abcam, USA. Goat anti-Rabbit IgG Secondary Antibody (HRP)-conjugated anti-rabbit secondary antibody (Item No. 6401-05) was obtained from Biovision, USA. Alexa Flour 488-labeled goat anti-rabbit IgG (H+L) (Item No. A0423) was purchased from Shanghai Beyotime Biotechnology. The first Strand cDNA Synthesis Kit (Item No. K1622) was obtained from Thermo Fisher Scientific, USA.

Major Instruments

iBright™ FL1000 imaging system and ProFlex™ PCR system were obtained from Thermo Fisher Scientific, USA. iMark microplate absorbance reader and FACSAria II Flow Cytometer were obtained from Bio-Rad, USA. FV1200 laser scanning microscope was obtained from OLYMPUS, Japan.

Methods

Cell Culture

The NCI-N87 cells were tested for contamination using the Mycoplasma test and found contamination-free. The cells were cultured in an RPMI-1640 medium containing 10% FBS and incubated at 37 °C with 5% CO_2. Cell morphology was observed using an inverted microscope, and subculture was carried out when the cell density reached over 80%, and the logarithm was taken. Cells in the growth phase were used for subsequent experiments. One day before transfection, cells were seeded into a 6-well plate and then divided into four groups: siRNA-negative control (siRNA-NC), siRNA-Enhancer of zeste homolog 2 (siRNA-EZH2), and siRNA-SUZ12 polycomb repressive complex 2 subunit (siRNA-SUZ12). The cells were transfected using the Lipofectamine 2000 reagent following the manufacturer's instructions. For subsequent experiments, the cells were collected after twenty-four hours of transfection.

Western Blot

Following two washes with PBS solution, the cells were centrifuged at 1500 rpm for 3 minutes, and the supernatant was discarded. A SDS-PAGE protein loading buffer was added followed by cell lysis on ice. After this, the cells were centrifuged at 15,000 rpm for 10 minutes and the supernatant was obtained. Subsequently, the proteins were quantified using the BCA method. The proteins were separated on SDS-PAGE and subsequently transferred onto the polyvinylidene fluoride (PVDF) membrane. The membranes were then blocked with 5% defatted milk powder. In the next step, the membranes were incubated overnight with Rabbit anti-human EZH2 and SUZ12 polyclonal antibodies (dilution ratio 1:1000) at 4 °C. However, β-actin was used as the internal reference. On the following day, the membranes underwent three rinses with TBST solution, each lasting 5 minutes. Subsequently, the membranes were incubated with HRP-labeled anti-rabbit (dilution ratio of 1:3000) secondary antibody at room temperature for 1 hour. The membranes were rinsed thrice with TBST solution, each for 5 minutes. Color development was achieved using the ECL method, and the bands were observed using a gel imaging system. Finally, a semi-quantitative analysis was conducted using Image J software.

qRT-PCR (Quantitative Real-time Reverse-transcription PCR)

The total RNA was extracted from cells using TRIzol method, and subsequently reverse transcribed into cDNA using First Strand cDNA Synthesis Kit (Item No. K1622, Thermo Fisher Scientific, USA). Primer 5.0 software was used for primer designing. The following primers used in qRT-PCR ((**Quantitative real-time reverse-transcription PCR)**) were:

EZH2: upstream primer 5′-TGTAAAACGACGGCCAGT-3′,

downstream primer 5′-AACAGCTATGACCATG-3′,

SUZ12: upstream primer 5′-AGGTCGACTCTAGAGG-3′,

downstream primer 5′- TCCTTGTAGTCCATCA-3′,

β-actin: upstream primer 5′-CCTAAGGCCAACCGTGAA-3′ and downstream primer 5′-GGAGCCAGGGCAGTA ATC-3′.

Furthermore, amplification conditions were set as follows: pre-denaturation at 95 °C for 30 seconds, followed by 40 cycles of 95 °C for 10 seconds, 60 °C for 10 seconds, 72 °C for 10 seconds, and the final extension at 72 °C for 10 minutes. The relative mRNA expression was assessed using the $2^{-\Delta\Delta Ct}$ method.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

The cells were seeded into a 96-well plate at a density of 5×10^3 cells/well. MTT solution was added to each well at 24, 48, and 72 hours followed by incubation at room temperature for 4 hours. Finally, optical density (OD) at 490 nm was assessed using enzyme-linked immunosorbent assay (ELISA), and the cell survival rate was calculated.

Transwell Assay

For cell migration, the cells were starved in a serum-free medium for 12–24 hours, and a Transwell chamber was placed on a 24-well plate. Pre-warmed serum-free medium was added into both the chamber and wells and subsequently incubated at 37 °C in the presence of 5% CO_2 for 2 hours. After this, the cells were digested with trypsin followed by centrifugation at 1000 rpm for 5 minutes, and then suspended in a serum-free medium. The cell concentration was adjusted to 2×10^5 cells/mL.

The upper chamber of the Transwell was filled with 500 µL of cell suspension, while the lower chamber was filled with 0.75 mL of the cell suspension supplemented with 10% FBS medium followed by incubation at 37 °C with 5% CO_2 for 24 hours. The upper chamber was gently swabbed with sterile cotton swabs, rinsed twice with PBS solution, and the residual liquid was discarded. Subsequently, 0.75 mL and 0.5 mL of 75% ethanol were added and rinsed twice with PBS solution. The cells were then stained with 0.75 mL and 0.5 mL crystal violet for 30 minutes. Finally, the cells were examined using a 100× microscope, 10 fields were randomly selected, and the mean number of migrating cells was determined.

For cell invasion analysis, 30 µL of Matrigel solution was diluted at 1:1 with serum-free Dulbecco's Modified Eagle Medium (DMEM) medium and subsequently added to the upper layer of the Transwell chamber (membrane pore size 12.0 µm). After this, the chamber was air dried at room temperature for 1 h. The remaining steps followed the cell migration outlined above. Finally, the mean number of the invading cells was calculated.

Flow Cytometry

The cells were rinsed twice with a pre-cooled PBS solution followed by the addition of 10 µL of Annexin V-FITC solution and 5 µL of the PI solution. Following a thorough mixing step, the cells were incubated in the dark for 10 minutes. The cells were then filtered using a 300-nylon mesh and subsequently processed for flow cytometry to assess cell apoptosis. Moreover, the FlowJo 10 software was used to perform data analysis and calculate the apoptosis rate.

Confocal Laser Scanning

For this purpose, the cells were adjusted to a concentration of 5×10^4 cells/mL, and subsequently inoculated on the slides. After this, the cells were fixed using 4% paraformaldehyde and permeabilized utilizing 0.5% Triton X-100 for 10 minutes. In the next step, the cells were blocked with 2% skim milk for 30 minutes followed by incubation with mouse anti-HUMAN H3K27me3 monoclonal antibodies at room temperature for 2 h. The cells underwent three PBS solution washes, each wash of 5 minutes. Furthermore, the cells were incubated with Alexa-Flour 488-labeled goat anti-rabbit IgG (H+L) antibody at room temperature for 1 h. The cells were then stained utilizing DAPI solution for 15 minutes and subsequently washed three times using PBS solution. Finally, using a confocal microscope, images at five random positions were captured, and fluorescence intensity was analyzed using the OLYMPUS CellSens software.

Co-IP Technique

Extracted proteins were cleaved using RIPA reagent, and subsequently incubated overnight with EZH2, SUZ12, and H3K27me3 antibodies at 4 °C. After this, pretreated hA agarose gel was added, and cells underwent a gentle shaking at 4 °C for 4 hours. After immunoprecipitation, the proteins were centrifuged at 12,000 rpm at 4 °C for 30 seconds. Furthermore, after discarding the supernatant, the pellet was rinsed thrice using lysis buffer. 1× SDS loading buffer was added and boiled for 5 minutes to dissolve the pellet. The proteins were separated on SDS-PAGE and subsequently transferred onto the PVDF membrane. The membrane was processed for the Western Blot analysis following the same steps as outlined in 1.2.2.

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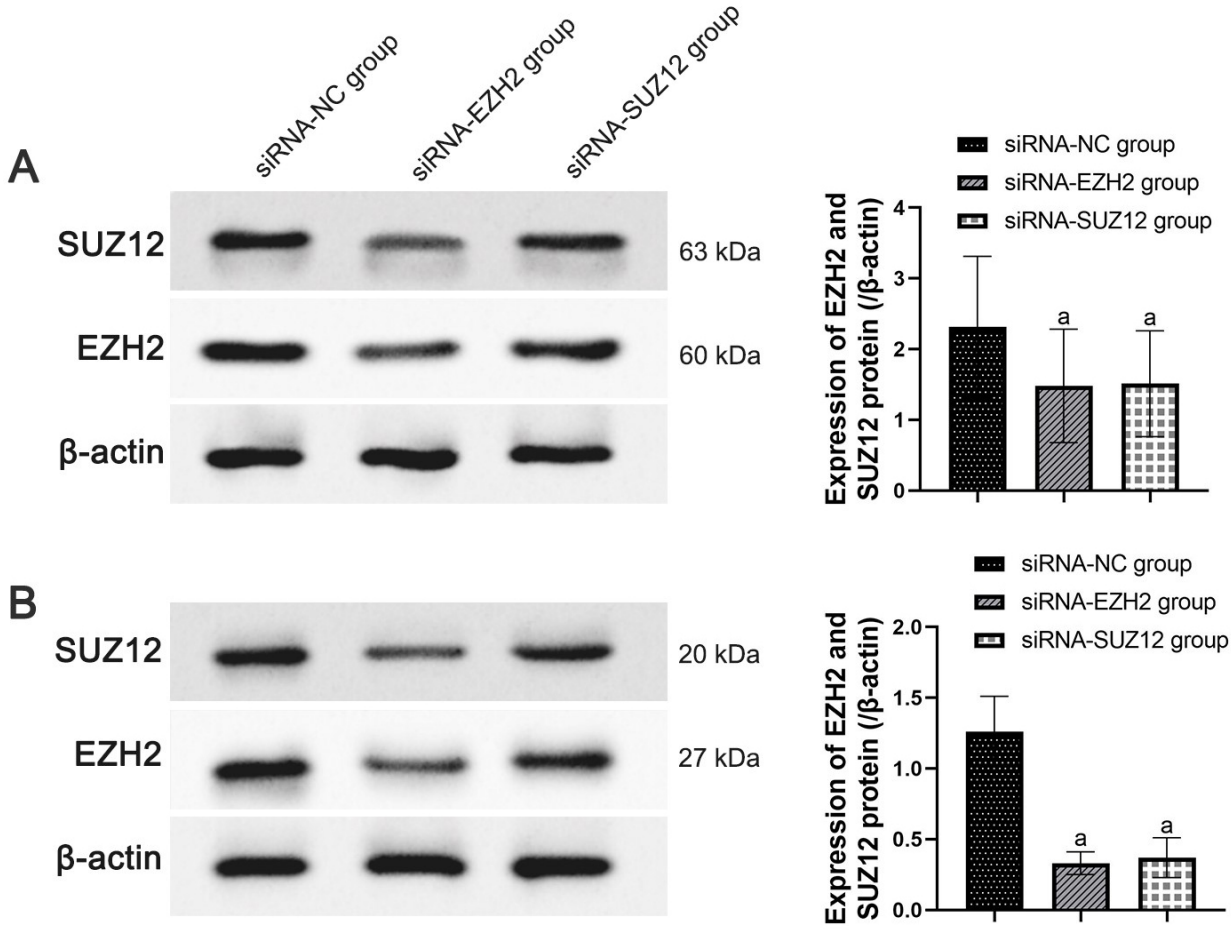

Fig. 1. Effect of siRNA lentivirus transfection on the expression of siRNA-Enhancer of zeste homolog 2 (siRNA-EZH2) (A) and siRNA-SUZ12 polycomb repressive complex 2 subunit (siRNA-SUZ12) (B) in gastric cancer cells. Note: Compared with the siRNA-negative control (siRNA-NC) group, $^{a}p < 0.05$.

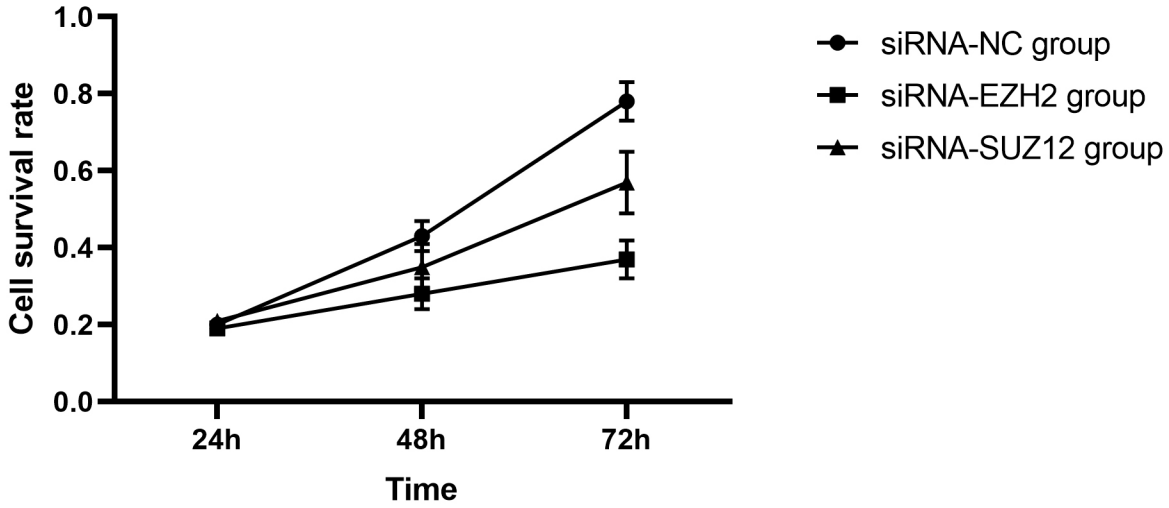

Fig. 2. Effects of down-regulation of EZH2 and SUZ12 on the proliferation of gastric cancer cells.

Statistical Analysis

The SPSS 20.0 software (IBM Corp., Armonk, NY, USA) was used for data analyses. For measurement data that followed a normal distribution, we used descriptive statistics to express the mean ± standard deviation. Inter-group comparisons were conducted using independent samples *t*-tests, while intra-group comparisons were assessed

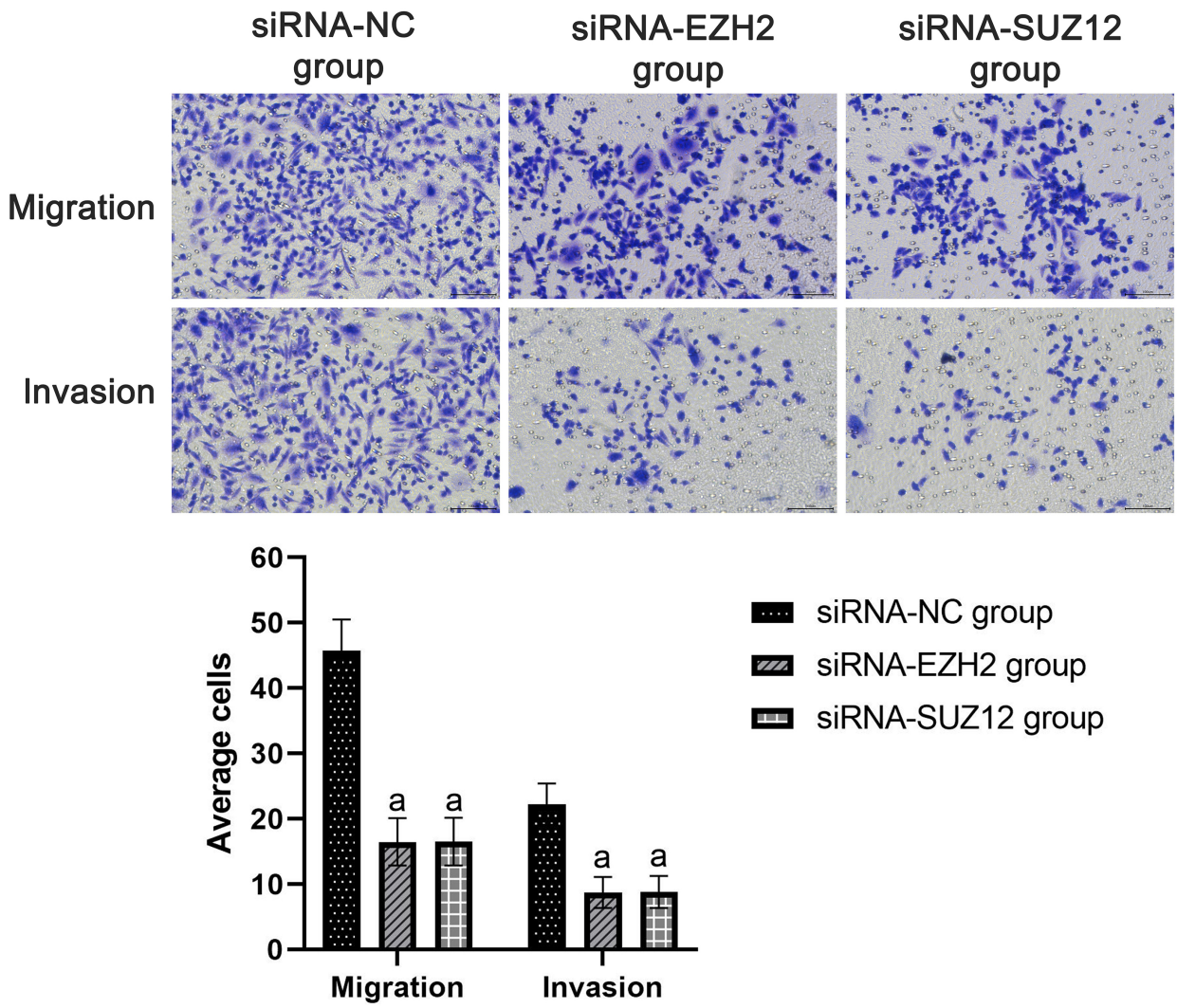

Fig. 3. Experimental results of migration and invasion of Transwell. Note: Compared with the siRNA-NC group, $^{a}p < 0.05$.

using paired-samples *t*-tests. Comparison among more than two groups was performed using one-way analysis of variance (ANOVA), and pairwise comparison was carried out using the Student-Newman-Keuls (SNK)-q post hoc test. However, measurement data that did not conform to a normal distribution were represented as median and interquartile range (M [P25, P75]). Two-group comparisons were performed using the Wilcoxon test, while three-group comparisons were conducted using the Kruskal-Wallis test. Enumeration data were presented as either frequencies or constituent ratios. Enumeration data were statistically analyzed using the chi-squared test. A *p*-value < 0.05 was considered statistically significant.

Results

Effects of siRNA Lentiviral Transfection on the Expression of EZH2 and SUZ12 in Gastric Cancer Cells

Western Blot analysis revealed significantly decreased concentrations of EZH2 protein and mRNA in the siRNA-EZH2 group and SUZ12 protein and mRNA in the siRNA-SUZ12 group as compared to the siRNA-NC group ($p < 0.0001$, Fig. 1).

Effects of Down-regulation of EZH2 and SUZ12 on the Proliferation of Gastric Cancer Cells

MTT findings revealed that compared with the siRNA-NC group, a significant reduction in cell viability was observed in both the siRNA-EZH2 group and the siRNA-SUZ12 group ($p < 0.0001$, Fig. 2).

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Table 1. Effects of down-regulation of EZH2 and SUZ12 on migration and invasion of gastric cancer cells.

Group	Average no. of migrated cells/cell	Average no. of invaded cells/unit
siRNA-NC group	45.79 ± 4.76	22.28 ± 3.16
siRNA-EZH2 group	16.48 ± 3.63[a]	8.74 ± 2.37[a]
siRNA-SUZ12 group	16.54 ± 3.65[a]	8.81 ± 2.44[a]
F-value	174.404	84.611
p-value	<0.0001	<0.0001

Note: Compared with the siRNA-NC group, [a]$p < 0.0001$

Effects of Down-regulation of EZH2 and SUZ12 on Migration and Invasion of Gastric Cancer Cells

Transwell's assay demonstrated that both the siRNA-EZH2 and siRNA-SUZ12 groups showed significantly reduced cell survival rates, mean number of migrating cells and invading cells compared to the siRNA-NC group ($p < 0.0001$, Table 1, Fig. 3).

Effects of Down-regulation of EZH2 and SUZ12 on Apoptosis of Gastric Cancer Cells

The apoptosis rate of the siRNA-EZH2 group and the siRNA-SUZ12 group was significantly elevated when compared with the siRNA-NC group ($p < 0.0001$, Fig. 4).

Effects of Down-regulation of EZH2 and SUZ12 on the Level of H3K27 Methylation Modification in Gastric Cancer Cells

The results of confocal laser scanning showed significantly elevated expression levels of H3K27me3 in the cells of the siRNA-EZH2 group and the siRNA-SUZ12 group compared to the siRNA-NC group ($p < 0.0001$, Fig. 5).

Interaction between PRC2 and H3K27 in Gastric Cancer Cells

Co-IP technology revealed an interaction between PRC2 and H3K27 in cells. Furthermore, EZH2 and SUZ12 were found to interact with H3K27me3 in gastric cancer cells (Fig. 6).

Discussion

Roughly half of the global incidence and deaths due to gastric cancer are attributed to China [7]. The onset of gastric cancer is linked to various contributing factors, and its insidious early symptoms often result in delayed diagnosis and consequently a poor prognosis [8]. Moreover, the limited treatment efficacy of gastric cancer is partially attributed to tumor metastasis despite surgical resection with curative intent, leading to frequent use of chemoradiotherapy. However, these non-surgical interventions are associated with potential collateral damage to adjacent tissues and cause toxic side effects. Therefore, there is an urgent need to seek a highly effective anti-gastric cancer drug that minimizes toxic side effects.

The epigenetic dysregulation of histone methylation has been an essential feature of malignant tumors and is involved in the development of tumors [9]. H3K27me3 is widely distributed on the entire genome and plays a role in the transcriptional repression of target genes [10]. Previous research has shown that PRC2, a highly conserved histone methyltransferase, interacts with H3K27, catalyzing the mono-, di- and tri-methylation of H3K27 [11]. It has been reported that the modification of H3K27me3 is a vital marker for gene silencing in epigenetics, which is involved in regulating the balance between cell differentiation and proliferation [12].

EZH2 and SUZ12 are core subunits of PRC2. Xu J *et al*. [13] reported that EZH2 in gastric cancer tissues was increased compared to normal tissues, and patients with highly expressed EZH2 exhibited a poor prognosis. Furthermore, the knockdown of EZH2 through siRNA inhibited gastric cancer cell proliferation and invasion. SUZ12 modulates various biological aspects of gastric cancer cells, including proliferation, metastasis, and invasion [14,15].

To further elucidate the role of the PRC2 complex in the development of gastric cancer, siRNA technology was used to knock down EZH2 and SUZ12 in gastric cancer cells. A significant reduction in SUZ12 protein and mRNA levels was observed in the SUZ12 group, confirming the successful transfection. Furthermore, the effect of downregulated EZH2 and SUZ12 levels on the biological behaviors of gastric cancer cells was evaluated using the MTT assay, Transwell method, and flow cytometry. The number of cells and invasive cells was remarkably reduced. In line with the results of previous studies, the apoptosis rate was significantly elevated, suggesting that downregulating the expressions of EZH2 and SUZ12 in gastric cancer cells can suppress cell proliferation, migration, and invasion, and offer a new target for treating gastric cancer [13,15]. Among the three primary types of H3K27 methylation, trimethylation is considered essential in the silencing mechanism of the PRC2 complex.

In our study, laser scanning confocal technology was used to assess the level of H3K27 methylation modification in gastric cancer cells and revealed significantly elevated expressions of H3K27me3 in both the siRNA-EZH2 and siRNA-SUZ12 groups compared to the siRNA-NC group, suggesting the downregulation of EZH2 and EZH2 in gastric cancer cells. These findings suggest that expression of

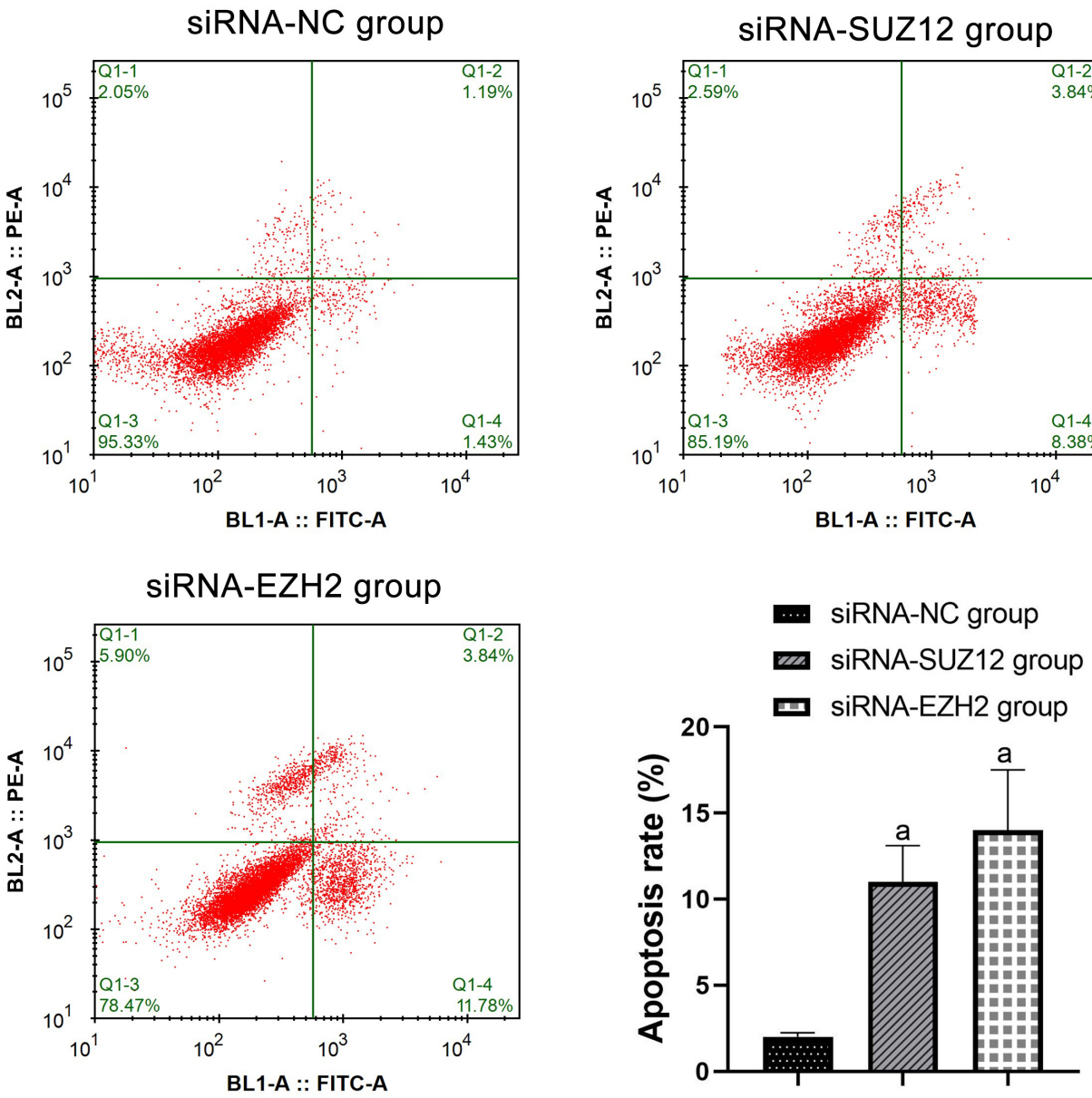

Fig. 4. Effects of down-regulation of EZH2 and SUZ12 on apoptosis of gastric cancer cells. Note: Compared with the siRNA-NC group, $^{a}p < 0.05$.

SUZ12 promotes the trimethylation of H3K27, which is involved in the tumorigenesis of gastric cancer. Co-IP is a classic technology using antibodies to capture target proteins and their interacting proteins and complexes, which can specifically enrich the target proteins. In this study, the CO-IP method was used to examine the interaction between PRC2 and H3K27 in gastric cancer cells. It was found that two core subunits of PRC2, EZH2 and SUZ12, interacted with H3K27me3, which was consistent with previous research [16]. These findings suggest that EZH2 and SUZ12 could serve as potential therapeutic targets for gastric cancer. Further studies need to compare the specific efficacy and molecular mechanism of these two targets.

Conclusion

In this study, we have explored the influence of the Polycomb Repressive Complex 2 (PRC2) on the behaviors of gastric cancer cells by regulating the methylation of H3K27me3. Our findings suggest that PRC2 plays a role in various aspects of gastric cancer cell biology, including proliferation, migration, invasion, and apoptosis. However, our study indicates an association between PRC2 and these cellular behaviors, it is important to acknowledge that our study did not encompass a comprehensive experimental validation to establish causal relationships.

The observed effects of PRC2 on gastric cancer cell behavior imply a potential regulatory role of this complex in

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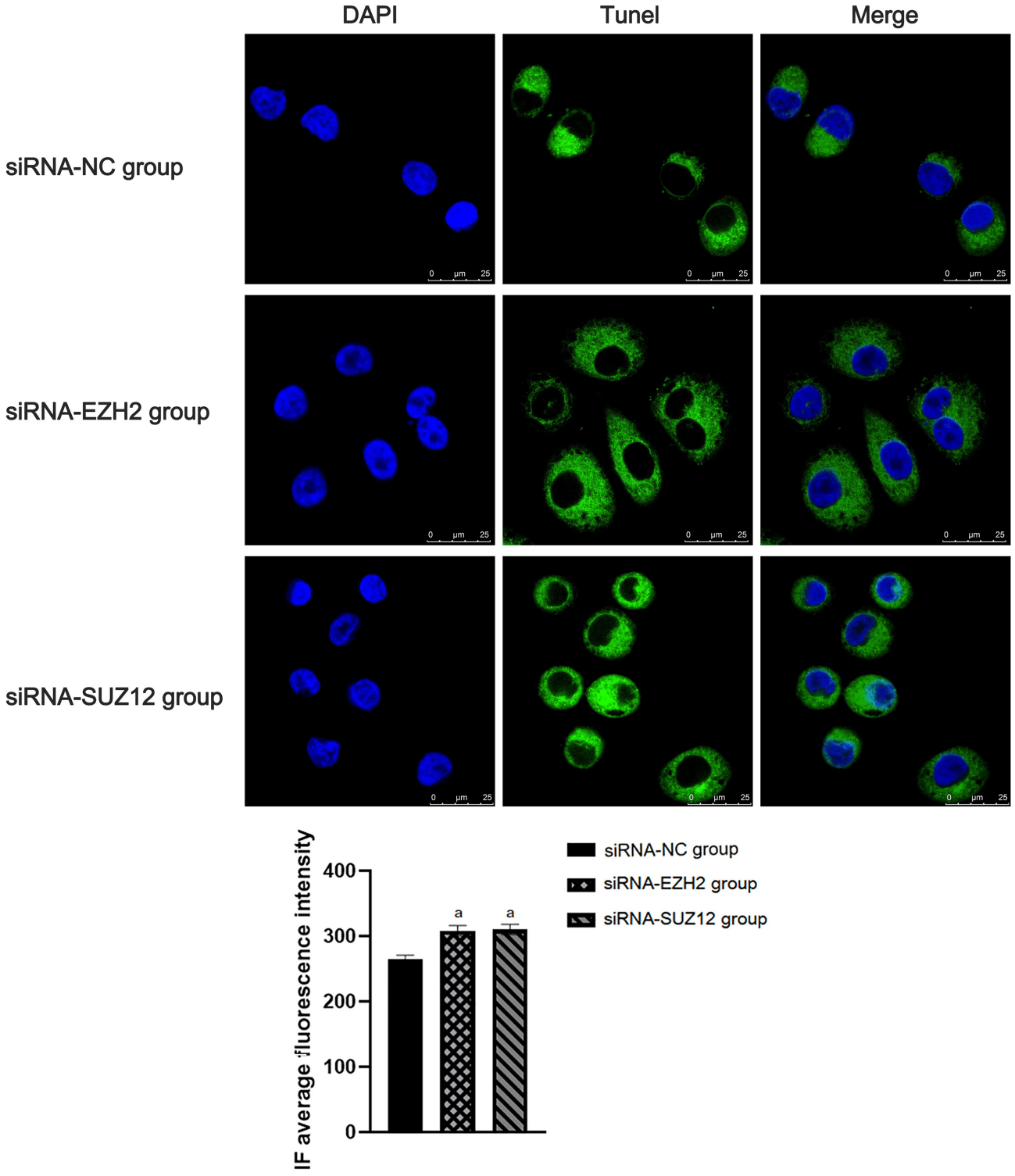

Fig. 5. Effects of down-regulation of EZH2 and SUZ12 on the level of Methylation of histone H3 on lysine 27 (H3K27) methylation modification in gastric cancer cells. Note: Compared with the siRNA-NC group, $^{a}p < 0.05$.

inhibiting proliferation, migration, and invasion while promoting apoptosis. However, it is crucial to recognize the limitations of this study. We did not conduct an entire array of experiments to conclusively establish the cause-and-effect relationship between PRC2, H3K27 methylation, and the observed cellular responses. Therefore, our study provides preliminary insights into the influence of PRC2 on gastric cancer cells and opens avenues for further investigation.

In the future, comprehensive experimental designs and functional assays are warranted to validate the proposed mechanisms and to elucidate the precise molecular path-

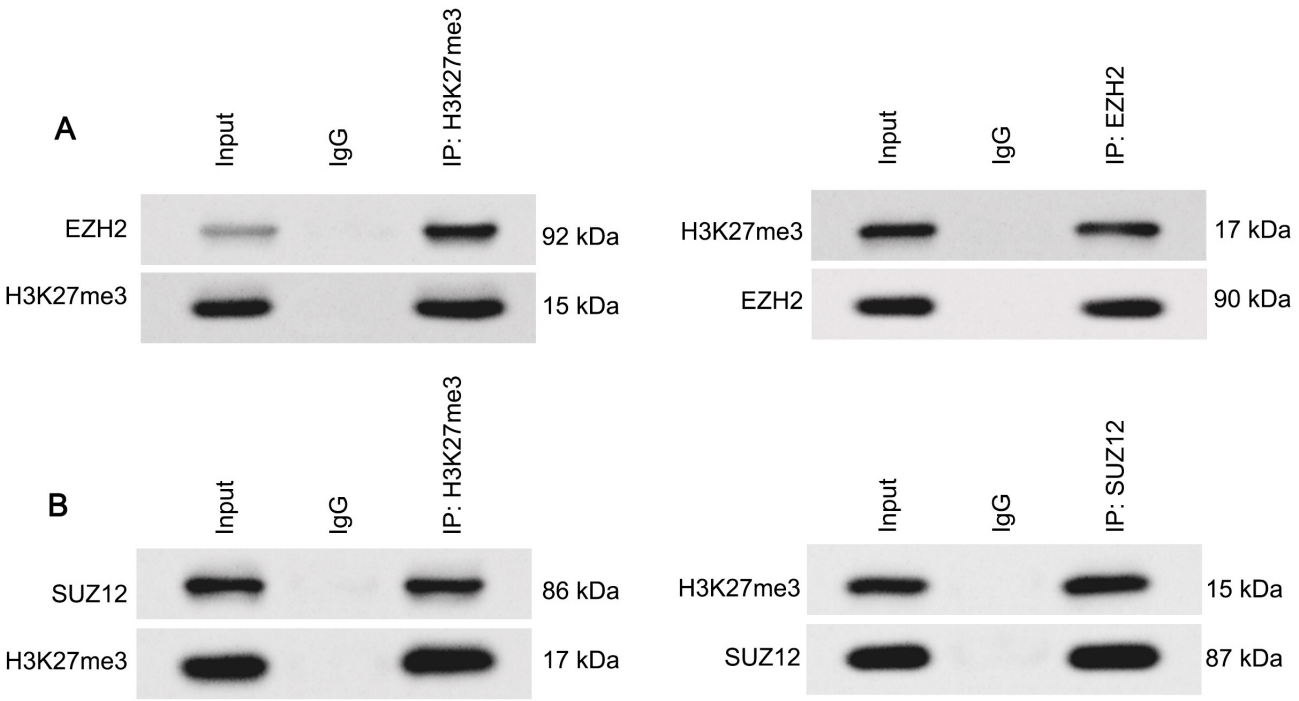

Fig. 6. Interaction between PRC2 and H3K27 in gastric cancer cells. The PRC2 (A) and H3K27 (B) expression was determined via WB.

ways influenced by PRC2 and H3K27 methylation in shaping gastric cancer cell behavior. These investigations will provide a more robust foundation for understanding the potential therapeutic implications of targeting PRC2 and epigenetic modifications concerning gastric cancer.

Availability of Data and Materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Author Contributions

CL designed the research study. XH performed the research. CL and XH conducted experiments, analyzed the data. Both authors contributed to editorial changes in the manuscript. Both authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

This study was approved by the ethics committee of The First Affiliated Hospital of Jinzhou Medical University, No.19759923. Informed consent was obtained from all study participants. All the methods were carried out in accordance with the Declaration of Helsinki.

Acknowledgment

Not applicable.

Funding

This research received no external funding.

Conflict of Interest

The authors declare no conflict of interest.

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