CRISPR/Cas9-mediated EZH2 knockout suppresses the proliferation and migration of triple-negative breast cancer cells

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Abstract. Triple-negative breast cancer (TNBC) is an aggressive subtype of BC characterized by extensive intratumoral heterogeneity. Compared with other types of BC, TNBC is more prone to invasion and metastasis. The aim of the present study was to determine whether adenovirus-mediated clustered regulatory interspaced short palindromic repeats (CRISPR)/Cas9 system is capable of effectively targeting enhancer of zeste homolog 2 (EZH2) in TNBC cells and lay an experimental basis for the investigation of the CRISPR/Cas9 system as a gene therapy for BC. In the present study, EZH2 was knocked out in MDA-MB-231 cells using the CRISPR/Cas9 gene editing tool to create EZH2-knockout (KO) group (EZH2-KO group). Moreover, the GFP knockout group (control group), and a blank group (Blank group), were employed. The success of vector construction and EZH2-KO were verified by T7 endonuclease I (T7EI) restriction enzyme digestion, mRNA detection and western blotting. Changes in proliferation and migration ability of MDA-MB-231 cells following gene editing were detected by MTT, wound healing, Transwell and *in vivo* tumor biology assays. As indicated by the results of mRNA and protein detection, the mRNA and protein expression of EZH2 were significantly downregulated in the EZH2-KO group. The difference in EZH2 mRNA and protein between the EZH2-KO and the two control groups was statistically significant. MTT, wound healing and transwell assay suggested that the proliferation and migration ability of MDA-MB-231 cells in the EZH2-KO group were significantly decreased after EZH2 knockout. *In vivo*, the tumor growth rate in the EZH2-KO group was significantly lower than that in the control groups. In brief, the present study revealed that the biological functions of tumor cells were inhibited after EZH2 knockout in MDA-MB-231 cells. The aforementioned findings suggested that EZH2 can have a key role in the development of TNBC.

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Introduction

Breast cancer (BC), with the highest incidence rate among types of female cancer, has been reported to be the second leading cause of death, jeopardizing the health of female patients (1). Triple-negative BC (TNBC) refers to a subtype of BC defined by the negative expression of estrogen and progesterone receptors, and the lack of amplification of the human epidermal growth factor-2 (2). Patients with TNBC have poor prognosis and a high risk of relapse and metastasis compared with patients with other subtypes due to the aggressive nature of TNBC, coupled with the lack of targeted therapies (3-5). Thus, effective TNBC therapies should be developed, and the design of novel therapeutic strategies for more effective disease management in TNBC has become a hotspot of research (6).

Clustered regulatory interspaced short palindromic repeats (CRISPR)/Cas9 system is emerging as a powerful tool for precision medicine (7,8). The CRISPR/Cas9 system, originally identified as an adaptive immune defense mechanism in bacteria, comprises two major components: Cas9 protein, as a nuclease, and the synthetic guide RNA (sgRNA). With the regulation of sgRNAs, Cas9 is recruited to the target site where it cuts DNA, allowing the removal/deletion and addition/insertion of DNA fragments. The CRISPR/Cas9 system has been successfully and effectively applied as a revolutionary, yet feasible, genome editing tool in rodents and embryonic stem cells and zygotes, as well as plants and other small experimental animals (9,10). Wang *et al* (11) adeptly utilized the CRISPR/Cas9 technology to generate mice with

multiple concurrent gene mutations, achieving remarkably high efficiency. In contrast to traditional techniques which require 2-3 years to produce a mouse carrying five gene mutations, this cutting-edge method accomplishes the same feat in a mere 2-3 weeks, and notably circumvents the need for embryonic stem cells.

Recent research has suggested that enhancer of zeste homolog 2 (EZH2) serves as a key component of polycomb repressive complex 2 (PRC2) (12). EZH2 serves a role in chromosomal structure formation, cell cycle regulation, senescence, cell differentiation and promotion of tumor growth and metastasis in malignant tumor models (13-15). Hussein *et al* (16) reported a notable correlation between high EZH2 expression and TNBC phenotype, suggesting its biological role in this aggressive disease. EZH2, serving as a potential oncogene in a wide variety of malignancies, has a marked role in promoting MMP activity and extracellular matrix degradation, indicating the role of EZH2 in BC metastasis (17). Accordingly, EZH2, a novel biomarker, may serve as a novel target for treating TNBC.

CRISPR/Cas9 system has been developed as a novel gene editing tool, capable of achieving high gene-targeted efficiency in different cell lines and small experimental animals, demonstrating that it is a promising gene therapy (18,19). To the best of our knowledge, however, the role of the CRISPR/Cas9 system in the editing of EZH2 in the treatment of BC remains unclear.

Effective targeted therapies for TNBC are limited, highlighting the urgent need for the development of novel therapeutic strategies. The CRISPR/Cas9 system has emerged as a powerful tool for gene editing and shows great promise in addressing the challenges posed by TNBC. By leveraging the precise and efficient gene editing capabilities of CRISPR/Cas9, we choose EZH2 as a targeting gene in TNBC, thereby providing valuable insight into the molecular mechanisms underlying TNBC pathogenesis.

Materials and methods

Chemicals and reagents. Table SI lists the chemicals and reagents used in the present study.

Cell culture. MDA-MB-231 (cat. no. TCHu227) and 293A (cat. no. SCSP-5094) cell lines purchased from the Cell Bank of the Chinese Academy of Sciences. MDA-MB-231 was cultured in L-15 medium and 293A was cultured in DMEM medium supplemented with 10% FBS (Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 mg/ml streptomycin at 37˚C with 5% CO_2.

gRNA design. The gRNA of EZH2 was designed online (http://crispor.tefor.net/; version 5.01) and that of green fluorescent protein (GFP) was designed as a control. EZH2 or GFP-specific gRNAs were designed using the N20NGG pattern. The N20NGG pattern allows for flexibility in selecting the nucleotides within the 20-base target region of the gRNA, enabling customization for specific target sequences. By utilizing this pattern, it increases the chances of finding suitable target sites within the desired gene or DNA sequence. The gRNA sequences and primers were synthesized by Sangon Biotech Co., Ltd. The parental vector, pENTRY-U6-hEF1a-Cas9 (cat. no. 111385; Addgene, Inc.), was used to construct the CRISPR vectors.

pENTRY-U6-hEF1a-Cas9 plasmid was digested with restriction endonuclease *Xma*I/*Pme*I and the digested product was purified. sgRNA oligos (Table SII) targeting the EZH2 or GFP genomic loci were annealed and ligated with linear pENTRY-U6-hEF1a-Cas9 using the Gibson enzyme. The following recombinant reaction procedure was used: A total of 0.5 μl pENTRY-U6-hEF1a-Cas9 plasmid (*Xma*I/*Pme*I double digestion), 2.0 μl sgRNA fragment and 2.5 μl Gibson enzyme were incubated at 50˚C for 1 h, transformed with *E. coli* and coated with Luria-Bertani (kanamycin-resistant plates). Next, monoclonal colonies were selected to extract plasmids for Sanger sequencing. The sequence was termed pENTRY-U6-EZH2-sgRNA-hEF1a-Cas9.

Adenovirus packaging. sgRNA expression vector pENTRY-U6-EZH2-sgRNA-hEF1a-Cas9 and the adenoviral backbone vector pAD-U6-EF1a-Cas9 were combined using Gateway technology to construct an adenoviral vector. To initiate the recombination process, 0.5 μl of the pENTRY-U6-EZH2-sgRNA-hEF1a-Cas9 plasmid and 0.5 μl of the pAD vector were mixed together. Then, 1 μl of the Gateway™ LR Clonase™ II enzyme mix (cat. no. 11791020; Thermo Fisher Scientific, Inc.), which contains the necessary recombinases, was added to the plasmid mixture. The plasmid mixture, along with the enzyme mix, was incubated for 1 h at 25˚C. During this incubation period, the recombinases facilitated the recombination of the pENTRY-U6-EZH2-sgRNA-hEF1a-Cas9 plasmid with the pAD vector. This recombination process involved specific DNA sequences within the plasmids, allowing for the insertion of the sgRNA expression cassette into the adenoviral backbone vector.

GFP and EZH2 adenoviral vectors were transformed, monoclonal colonies were selected by kanamycin and plasmid was extracted and then identified with the *Xba*1 restriction enzyme; the recombinant gRNA vectors were termed EZH2-pAD-Cas9 and GFP-pAD-Cas9, respectively. The recombinant plasmid was linearized with *Pac*I enzyme, and the linearized pAD vector was transfected into 293A cells using Lipofectamine® 3000 (Cat. No. L3000015; Thermo Fisher Scientific, Inc.). The virus was collected when cells were lesioned. Viral particles were purified by cesium chloride gradient centrifugation (8,000 x g, 5 min), which yielded EZH2 and GFP adenoviruses. The viral titer was determined based on the endpoint dilution method (20). The formula was used under the following conditions: A^-, the negative control, did not have CPE and proliferation inhibition, while B^-, the minimum dilution of the crude virus extracts, resulted in CPE.

Transduction of CRISPR adenovirus. MDA-MB-231 cells were seeded in six-well plates at a density of $1.5x10^6$ cells/well 1 day prior to transduction and grown to 70% confluence. MDA-MB-231 cells were transfected with EZH2 adenovirus or a GFP adenovirus. According to the cell MOI and virus titer, the corresponding virus volume was added, as determined using the following formula: Virus volume=(MOI x number of cells)/virus titer (MOI of MDA-MB-231: 10). At 48 h

post-transduction at 37˚C by puromycin (2.00 μg/ml), MDA-MB-231 cells were harvested, and genomic DNA was extracted for PCR and T7 endonuclease I (T7EI) digestion. The T7EI editing assay was performed as previously reported (21). With MDA-MB-231 cell genomic DNA as a template and T7EI sense and antisense primers, a reaction mixture was prepared containing 5 μl of PCR product (300 ng), 3 μl of distilled water and 1 μl of 10xT7 buffer, resulting in a total volume of 9 μl. An annealing program was performed with an initial temperature of 95˚C for 5 min, followed by a gradual decrease of 2˚C per second until reaching 85˚C, and then decrease at a rate of 0.5˚C per second until reaching 16˚C. After completion of the annealing step, 1 μl of T7E1 enzyme was added to the annealed product, the contents were thoroughly mixed and incubate at 37˚C for 20 min to allow for the digestion reaction to occur. Upon completion of the reaction, 1.5 μl of 0.25 mol/l EDTA was added to stop the digestion reaction.

Reverse transcription-quantitative PCR (RT-qPCR). At 48 h after EZH2 adenovirus infection, cells were collected and total RNA was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA was reverse-transcribed into cDNA using SuperScript™ III First-Strand Synthesis System (cat. no. 12574018; Thermo Fisher Scientific, Inc.). Human GAPDH was used as an internal loading control for qPCR. EZH2 was amplified using cDNA as a template to detect expression of EZH2 mRNA in MDA-MB-231 cells following EZH2 adenovirus infection. qPCR was performed using reagents and protocols from the Applied Biosystems SYBR Green Master Mix Kit (cat. no. A46012; Thermo Fisher Scientific, Inc.). qPCRs were performed using a Roter-Gene 300 thermal cycling instrument (Corbett Life Science). The following thermocycling conditions were used for qPCR: Initial denaturation at 95˚C for 2 min, followed by 38 cycles of denaturation at 95˚C for 15 sec, and annealing and elongation at 60˚C for 30 sec. The primers used to amplify EZH2 and GAPDH are provided in Table SII. The relative expression of EZH2 mRNA was calculated using the $2^{-\Delta\Delta Cq}$ method (22).

Agarose gel electrophoresis. MDA-MB-231 cells were transfected with EZH2 adenovirus or a GFP adenovirus. At 48 h post-transduction, MDA-MB-231 cells were harvested. Genomic DNA was extracted from cells and EZH2 loci were analyzed using the T7EI assay. T7EI recognizes and cleaves the mismatched DNA structure, resulting in a short strand cleaved by T7EI. The DNA sample was resuspended in an appropriate buffer and transferred to a PCR tube. The sample was then heated to 68˚C for 15 min, then 95˚C for 10 min in a thermocycler. The temperature was then reduced to 4˚C. 2 μl of the resuspended sample was used as a template for the PCR. The primers used for this PCR were EZH2 sense primer and EZH2 antisense primer. The PCR was then conducted with the following steps: Denaturation at 95˚C for 30 sec to separate the double-stranded DNA into single strands. Annealing at 57˚C for 30 sec for the primers to bind to the DNA template. Extension at 72˚C for 40 sec for the Taq polymerase to replicate the DNA. These cycles were repeated 40 times. After the PCR was complete, the amplified DNA fragments were run on a 2% agarose gel in 1X TBE buffer.

Western blot analysis. When MDA-MB-231 cells reached 70-80% confluence, they were collected and lysed with RIPA (cat. no. P8340; Merck KGaA) at 72 h after EZH2 adenovirus infection, and total protein was extracted. Protein concentration was quantified with a BCA kit and then detected by western blotting. A total of 50 μg protein/lane was separated by 10% SDS-PAGE, transferred to PVDF membranes (cat. no. 03010040001; Roche) and blocked with 5% skimmed milk at 25˚C for 1 h. Rabbit anti-human EZH2 polyclonal antibody (cat. no. ab186006; 1:1,000 dilution; Abcam) and mouse anti-human anti-GAPDH antibody (cat. no. D190090; 1:3,000 dilution; Sangon Biotech) were added and membranes were incubated overnight at 4˚C. Subsequently, the PVDF membranes were washed three times with 20% Tween-20 in tris-buffered saline (TBST) and incubated with horseradish peroxidase-labeled goat anti-rabbit secondary antibody or goat anti-mouse secondary antibody (1:5,000) for 2 h at 25˚C. Next, the membrane was washed three times with TBST. Protein bands were visualized using ECL. Performing grayscale analysis of Western Blot bands using ImageJ (version: 1.54D; National Institutes of Health).

MTT assay. Prepare a working solution of MTT by dissolving MTT powder in 0.01M PBS to the desired concentration (0.5 mg/ml). MDA-MB-231 cells at the logarithmic proliferation phase were seeded in 96-well plates (2,000/well). The adenovirus solution was added when the cell density was 70-90%. The MTT assay was performed at 24, 48, 72 and 96 h following infection of MDA-MB-231 cells with the adenovirus. The cells were assigned to three groups: EZH2-KO group, Control group and Blank group. The optical density was examined at 490 nm using a microplate reader. A cell proliferation curve was plotted.

Transwell assay. MDA-MB-231 cells were divided into EZH2-KO group, Control group and Blank group. A total of 500 μl/well L-15 medium supplemented with 20% FBS was added into a 24-well plate. The Transwell chamber was placed in a 24-well plate. The upper chamber was inoculated with 200 μl ($2x10^5$) MDA-MB-231 suspension supplemented in serum-free medium after 48 h of adenovirus infection. Following 24 h cell culture at 37˚C, the chamber was removed and the cells in the upper chamber that did not pass through the chamber membrane were removed with a cotton swab. The cells were fixed with 4% paraformaldehyde for 10-15 min and stained with 0.1% crystal violet solution at ambient temperature. After 30 min, samples were rinsed with water and then dried. A total of five randomly selected fields of view were observed under a light microscope (x100 magnification), and the number of cells passing through the Transwell cell polycarbonate membrane was counted manually. The experiment was performed in triplicate.

Would-healing assay. A total of $2x10^5$ MDA-MB-231 cells from the EZH2-KO Group, Control Group and Blank Group were placed in a 24-well plate. Then, a 200-microlitre sterile pipette tip was used to draw a line across the central area of cell proliferation, after cell debris was washed away with PBS, the cells were cultured with 5% FBS at 37 ˚C and captured at 0, 12, 24 and 48 h under a light microscope (x200 magnification).

In vivo tumor biology. The animal experiments were approved by the Ethics Review Committee of Guangxi University of Chinese Medicine. NOD/SCID, 6-8-week-old mice, female, average weight of 25 g) purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). They were housed under a 12-h light/dark cycle. In brief, $2x10^6$ MDA-MB-231 cells from EZH2-KO, control and blank groups in 0.2 ml PBS were inoculated subcutaneously in the right flank each mouse (n=3 mice per group). Tumor volume was evaluated with a slide caliper and determined by the following formula: V=length x $width^2/2(mm^3)$. These examinations were conducted every 5 days, which ensured accurate tracking of ulceration progression without causing undue disruption to the typical behavior of animals or imposing unnecessary stress. At 25 days after inoculation, all mice were anaesthetized with intramuscular injection of 50 mg/kg ketamine mixed with 5 mg/kg xylazine (23). Mice were euthanized by cervical dislocation and the tumor volume was examined.

Statistical analysis. All statistical analyses were performed using GraphPad Prism 9.5.1 software (Dotmatics). All quantitative data from at least three independent experiments are expressed as the mean ± SD. Statistical analysis of mRNA expression was performed using one-way ANOVA with Bonferroni's post-hoc test to evaluate differences between three groups. Statistical analysis of the proliferation rate, migration ability and tumor volume were performed using Kruskal-Wallis followed by Dunn's multiple-comparisons test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Virus preparation and confirmation of in vitro CRISPR/Cas9 system knockout of EZH2 gene. The recombinant pAD plasmid linearized with *Pac*I was transfected into 293A cells for 7-9 days and CPE was identified (Fig. 1A).

Before investigation of the *in vitro* and *in vivo* EZH2-silencing effect of the CRISPR/Cas9 system to generate EZH2 loss-of-function, the editing efficiency of EZH2-sgRNA for EZH2 was tested in MDA-MB-231 cells. After MDA-MB-231 cells were infected with the EZH2 adenovirus for 48 h, genomic DNA was extracted from cells and EZH2 loci were analyzed using the T7EI assay. T7EI recognizes and cleaves the mismatched DNA structure, resulting in a short strand cleaved by T7EI (Fig. 1B). Thus, two fragments were generated by T7EI digestion (Fig. 1B).

EZH2 expression is decreased at both the mRNA and protein levels. Following adenovirus infection of MDA-MB-231 cells, the mRNA expression of EZH2 in the EZH2-KO group was 0.58±0.06, which was significantly lower than in the control group (0.92±0.02) and in the blank group (0.846±0.035) (Fig. 2A).

Western blotting was performed 72 h after adenovirus infection of MDA-MB-231 cells. EZH2 protein expression in MDA-MB-231 cells infected with EZH2 adenovirus was downregulated compared with that in control and blank groups (Fig. 2B). However, the expression of EZH2 in the control and blank groups was similar.

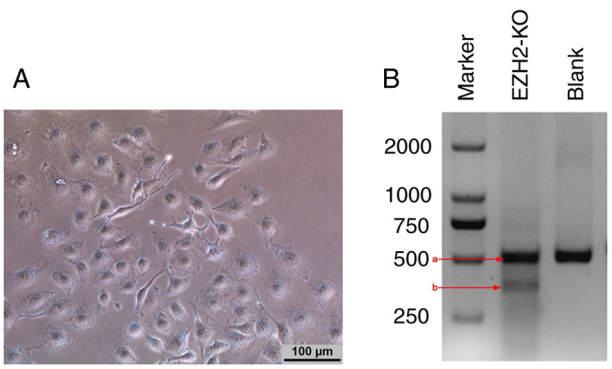

Figure 1. The EZH2 gene wis knocked out via the CRISPR/Cas9 system *in vitro*. (A) Edited 293A cells. Recombinant pAD plasmid linearized with *Pac*I was transfected into 293A cells for 7-9 days. Scale bar, 100 μm. (B) Agarose gel electrophoresis. M, marker; lane 1, PCR product of the EZH2-knockout group. lane 2, blank control. EZH2, enhancer of zeste homolog 2; KO, knockout.

KO of EZH2 decreases proliferative and migratory abilities of MDA-MD-231 cells. After MDA-MB-231 cells were infected with the adenovirus for 24 h, the proliferation rate of the cells was decreased. With the extension of the infection time, the proliferation rate of MDA-MB-231 cells was significantly lower than that of the control and blank groups (Fig. 3A). Compared with the control and blank groups, the proliferation rate of MDA-MB-231 cells in the EZH2-KO group declined after 48, 72 and 96 h, and the difference was statistically significant at 96 h ($P<0.05$).

Wound healing assay suggested that the would-healing area in EZH2-KO group was smaller compared with that in control and blank groups (Fig. 3B and C). Transwell assay showed the number of MDA-MB-231 having migrated through the transwell, and the lower layer of the filter decreased in EZH2-KO group (Fig. 3D and E). these results suggest that inhibition of EZH2 expression decreased the migration ability of MDA-MB-231 cells.

KO of EZH2 decreases tumor growth in vivo. Smaller tumor size wase found in the *EZH2*-KO group compared with the control and blank groups (Fig. 4A). The tumor volume in the EZH2-KO group was smaller in than that in the control and blank groups (Fig. 4B). The aforementioned results suggest that KO of *EZH2* suppressed tumor proliferation *in vivo*.

Discussion

The development of TNBC, a common malignant tumor in female patients, has been reported as a complex process of genetic and epigenetic changes (24). Epigenetics is a hereditary gene expression regulation mode that involves DNA sequence changes, including histone modification, gene imprinting and DNA methylation (25).

Introducing a foreign gene into cells of an organism and achieving efficient expression or silencing of an endogenous gene are the goals of gene therapy (26). With the advance of tumor therapy, gene therapy has become a research topic in cancer treatment. Notably, inhibition of oncogene expression has an inhibitory effect on tumor growth due to the

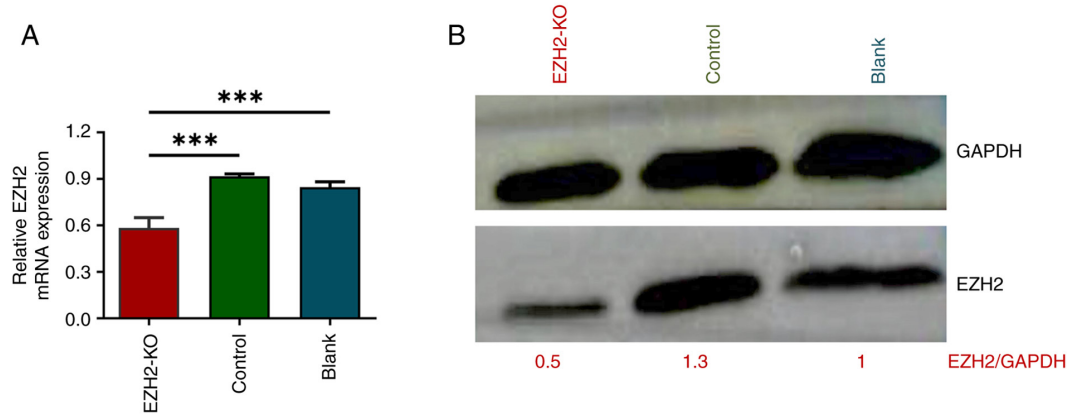

Figure 2. EZH2 expression is modulated at both the mRNA and protein levels. (A) EZH2 mRNA levels were analyzed by reverse transcription-quantitative PCR experiments in MDA-MB-231 cells treated by EZH2-KO group, Control group and Blank Group. (B) EZH2 protein levels were analyzed by western blot experiments in MDA-MB-231 cells treated by EZH2 EZH2-KO group, Control group and Blank Group. Quantification is shown below each blot (signal normalized to GAPDH, calculated as a fold change compared to Blank group, quantification by imageJ). ***P<0.001. EZH2, enhancer of zeste homolog 2; KO, knockout.

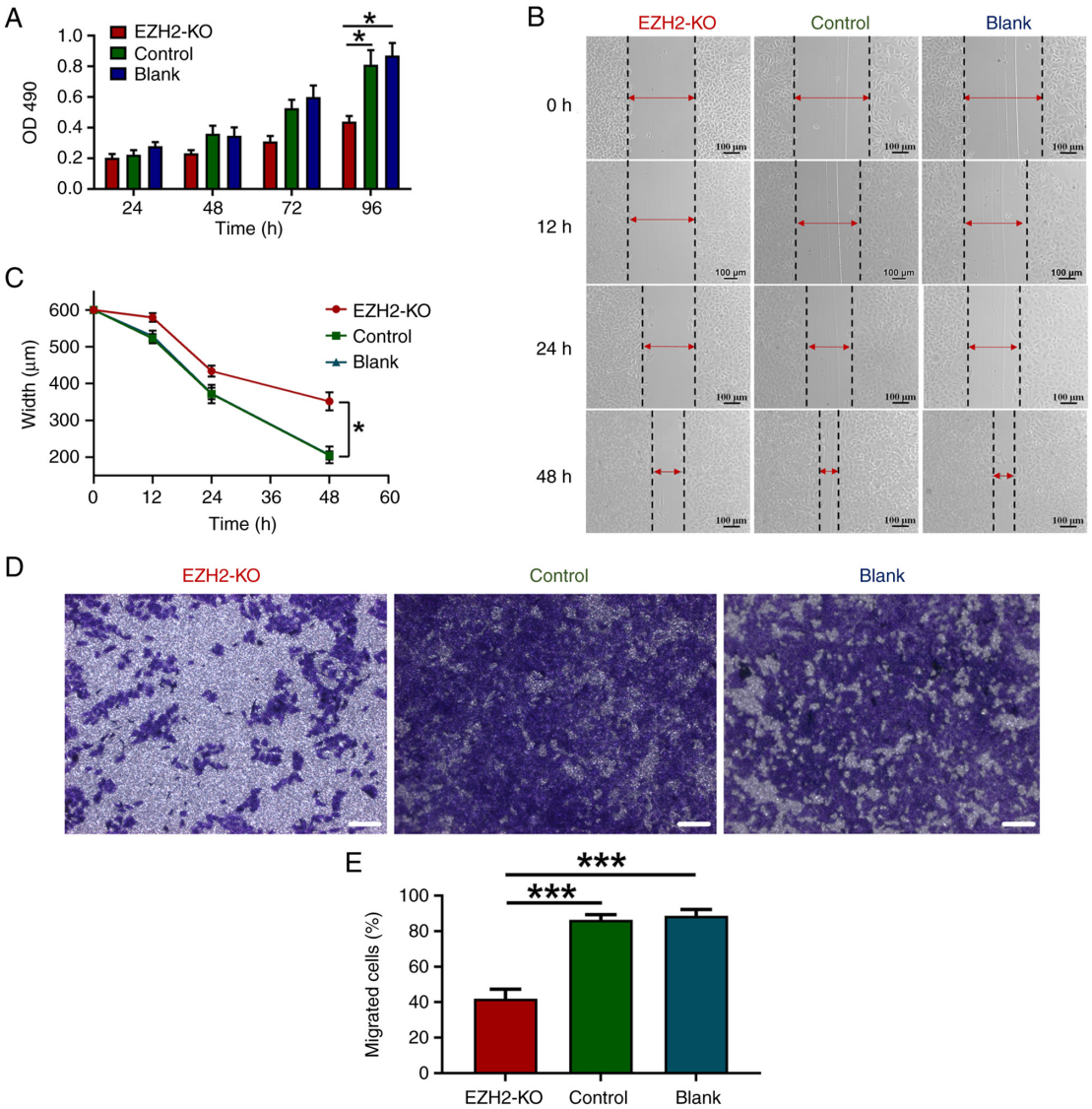

Figure 3. KO of EZH2 decreases proliferative and migratory abilities of MDA-MB-231 cells. (A) The MTT assay detect the proliferation of MDA-MB231 cells treated with EZH2 adenovirus infection (EZH2-KO Group), GFP adenovirus infection (Control group) and Blank group. (B) Migration effects of MDA-MB-231 cells treated with EZH2 adenovirus infection (EZH2-KO group), Green fluorescence protein adenovirus infection (Control group) and Blank group were examined by transwell migration assay. The scale bar represents 100 μm. (C) The histogram of the Transwell assay results. (D) The wound healing assay was performed to detect cell migration. The scale bar represents 100 μm. (E) The quantitative analysis of wound healing assay. *P<0.05, ***P<0.001. EZH2, enhancer of zeste homolog 2; KO, knockout; OD, optical density.

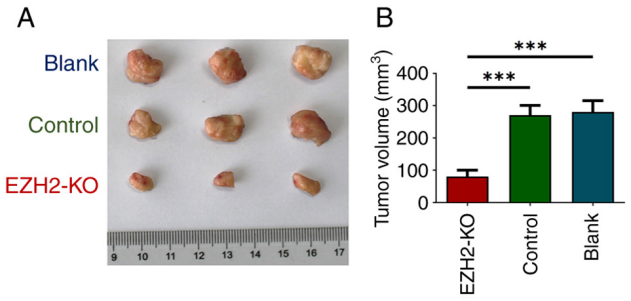

Figure 4. KO of EZH2 decreases triple-negative breast cancer tumor growth *in vivo*. (A) MDA-MB-231 cells were subcutaneously implanted into mice and harvested 25 days after inoculation. Smaller tumors were observed in the EZH2-KO compared with control and blank groups. (B) Differences in tumor volumes between the EZH2-KO and the control and blank groups were statistically significant. ***P<0.001. EZH2, enhancer of zeste homolog 2; KO, knockout.

overexpression of certain dominant oncogenes (27). Selecting optimal gene therapy technology to inhibit oncogene expression is a key step in gene therapy (28,29). At present, the CRISPR/Cas9 technology is widely used to establish cell and animal models related to tumor genes (30). Thus, the adenovirus system was used in the present study to mediate CRISPR/Cas9-targeted KO of EZH2.

As a defense mechanism, prokaryotes developed an adaptive immune system, termed CRISPR (31). The role of the short repeat sequences remained unclear until 2005 when several groups described similarities between the aforementioned sequences and phage DNA, raising the hypothesis that the aforementioned sequences are part of an adaptive immune system in bacteria (32,33). In 2013, type II Cas9 protein from *Streptococcus pyogenes* was first used for RNA-guided DNA cleavage in mammalian cells, providing a basis for using CRISPR/Cas9 as a genome editing tool (34). CRISPR/Cas9 technology is applied to explore the role of specific genes in cancer development, which may lead to the discovery of novel oncogenes or tumor suppressor genes (30). Feng *et al* (35) knocked down CDK11 in osteosarcoma cell lines based on the CRISPR/Cas9 system; cell viability, proliferation, migration and invasiveness were significantly decreased following knockout, and CDK11 may serve as a novel target for osteosarcoma therapy.

EZH2 is a key member of the PRC2 silencing complex that exhibits methyltransferase activity and catalyzes histone H3 on lysine 27 trimethylation and heterochromatin formation, and thereby mediates tumor suppressor gene expression silencing (36). The overexpression of EZH2 has been identified in prostate and bladder cancer, melanoma and TNBC (37-40). Moreover, extensive research has suggested that EZH2 overexpression is associated with poor prognosis in numerous types of cancer (41-43).

In the present study, the CRISPR/Cas9 gene editing technology was used. mRNA and protein levels of EZH2 tended to decrease after MDA-MB-231 cells were infected with the adenovirus. CRISPR/Cas9 system targeting EZH2 was found to inhibit EZH2 mRNA and protein expression in MDA-MB-231 cells. Functional assays confirmed that knocking out EZH2 inhibited proliferation and migration of MDA-MB-231 *in vitro*. Finally, the effect of EZH2 KO *in vivo* was validated, knockout of EZH2 gene expression inhibited tumor progression.

In the present study, a basis was provided for clinical application of gene therapy for cancer using CRISPR/Cas9 adenovirus system. Monoclonal cells in which the EZH2 was knocked out were selected using the *in vitro* dilution method (17) and the functional role of EZH2 in TNBC was examined. In future, the interaction of EZH2 with certain signaling pathways, oncogenes or tumor suppressor genes should be explored to gain more insight into the effects of EZH2 on tumorigenesis and its role in the pathogenesis of tumors.

While the present study provides key insight into the impact of gene therapy based on the CRISPR/Cas9 adenovirus system, findings are based on experiments conducted in a single cell line. Therefore, the extent to which these results can be generalized to other cell lines or *in vivo* contexts remains uncertain. Future studies should aim to validate and extend the findings of the current study in different cell lines and more complex biological systems.

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Availability of data and materials

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

Authors' contributions

QQM and LY conceived and designed the study and methodology. QQM, PW, HL, XLF and XCG performed the experiments and acquired the data. PW, HL, XLF and XCG analyzed and interpreted the data. QQM, HL and LY wrote, reviewed and revised the manuscript. LY provided study supervision. All authors have read and approved the final manuscript. QQM and LY confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The Ethics Committee of The First Affiliated Hospital of Guangxi University of Chinese Medicine approved the present study. The animal experiments were approved by the Ethics Committee of Guangxi University of Chinese Medicine (approval no. 20230214N1).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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