Truncated Bid Overexpression Induced by Recombinant Adenovirus Cre/LoxP System Suppresses the Tumorigenic Potential of CD133+ Ovarian Cancer Stem Cells

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Ovarian cancer is one of the most lethal malignant gynecologic tumors with a high relapse rate worldwide. Cancer stem cells (CSCs) have been identified in ovarian cancer and other malignant tumors as a small population of cells that are capable of self-renewal and multidifferentiation. CD133+ ovarian CSCs have been reported to be more tumorigenic and more resistant to chemotherapeutic treatment. Thus, CD133 has emerged as one of the most promising therapeutic markers for ovarian cancer treatment. In the current study, we constructed a recombinant adenovirus Cre/loxP regulation system to selectively introduce truncated Bid (tBid) expression specifically targeting CD133+ in ovarian CSCs. The results demonstrated that the coinfection of Ad-CD133-Cre and Ad-CMV-LoxP-Neo-LoxP-tBid significantly increased tBid expression in CD133+ ovarian CSCs. Moreover, the tBid overexpression induced by a recombinant adenovirus Cre/loxP system dramatically inhibited cell proliferation and invasion, significantly elevated cell apoptosis, and activated the mitochondrial apoptosis pathway in CD133+ ovarian CSCs. Additionally, recombinant adenovirus Cre/loxP system-mediated tBid overexpression suppressed the tumorigenic potential of CD133+ ovarian CSCs in a xenograft mouse model. In conclusion, our study successfully constructed a recombinant adenovirus Cre/loxP system and induced tBid overexpression in CD133+ ovarian CSCs, providing a new therapeutic approach for ovarian cancer treatment.

Key words: Truncated Bid (tBid); Cre/loxP system; Ovarian cancer; CD133; Cancer stem cells (CSCs)

INTRODUCTION

Ovarian cancer, one of the most lethal malignant gynecologic tumors, ranks as the fifth most common cause of cancer-related deaths, with 6.6 new cases per 100,000 women occurring worldwide each year1. Early-stage ovarian cancer can be successfully removed by surgery alone, but approximately 70% of patients are diagnosed with ovarian cancer at either stage III or IV2. At the advanced stages of ovarian cancer, surgical resection followed by platinum-based chemotherapy has been considered to be the standard treatment. However, most patients ultimately develop recurrence after treatment, which causes insurmountable obstacles for improving the survival rate3,4. Despite the advances in surgical method and chemotherapy agents, durable cures have not significantly increased in ovarian cancer treatment. Thus, it is urgent to develop effective treatment strategies to improve disease survival.

Cancer stem cells (CSCs; also called initiating cancer cells), a small subpopulation of cancer cells that possess multidifferentiation and self-renewal capacities, have been reported to be essential in cancer progression5. Populations of CSCs have been shown to be more tumorigenic in multiple experimental systems, including increased tumorigenicity, invasion, metastasis, angiogenic stimulation, and resistance to therapy6–9. It has been reported that CSCs were defined based on differential expression of cell surface markers (such as
CD133+ cells were dissociated with 0.25% trypsin (Sigma-Aldrich) and resuspended with ice-cold phosphate-buffered saline (PBS). The single cells were incubated with anti-CD133 monoclonal antibody-labeled microbeads (Miltenyi Biotech, Auburn, CA, USA) for 20 min at 4°C in the dark. Then samples were washed with ice-cold PBS and sorted on a BD fluorescence-activated cell sorting (FACS) Aria system (BD Biosciences, San Jose, CA, USA). The sorted CD133+ cells were cultured in a serum-free medium supplemented with 20 ng/ml human recombinant epidermal growth factor and 10 ng/ml human recombinant basic fibroblast growth factor (Invitrogen, Carlsbad, CA, USA).

**Flow Cytometric Analysis**

The purity of sorted CD133+ was detected using flow cytometric analysis. Briefly, cells were dissociated with trypsin, washed with ice-cold PBS, and resuspended into single-cell suspensions. Then cells were stained and incubated with mouse anti-human phycoerythrin (PE)-labeled CD133 (Miltenyi Biotech) for 30 min at 4°C in the dark. After incubation, the cells were washed with PBS and analyzed by FACS.

**Construction, Production, and Purification of Recombinant Adenovirus**

The cDNA fragments of the CD133 promoter and Cre were amplified and inserted into adenovirus pShuttle plasmid (Stratagene, Santa Clara, CA, USA) to construct the Ad-CD133-Cre recombinant adenovirus. The cDNA fragments of LoxP-Neo-LoxP and tBid were inserted into pShuttle-CMV plasmid (Stratagene) to construct the Ad-CMV-LoxP-Neo-LoxP-tBid recombinant adenovirus. The recombinant pShuttle plasmids and pAdEasy-1 plasmids (Stratagene) were homologously recombined in BJ5183 bacteria. The recombinated plasmids were linearized and transfected into 293T cells (ATCC) to produce the recombinant virus. After 14 days, cells were harvested and lysed by freeze–thawing. The supernatants were collected and concentrated by CsCl gradient centrifugation. The titers were determined using the 50% tissue culture infectious dose method. CD133+ cells were infected with Ad-CD133-Cre at 20 multiplicity of infection (MOI) and Ad-CMV-LoxP-Neo-LoxP-tBid at 30 MOI.

**Luciferase Reporter Assay**

Sorted CD133+ cells were seeded in 24-well plates. Twenty-four hours later, indicated luciferase reporter plasmids plus 3 ng of pRL-TK Renilla plasmid were transfected into the cells using Lipofectamine 2000 reagent (Life Technologies, Carlsbad, CA, USA) for 48 h. The luciferase activity was detected by a fluorescence detector (Promega, Madison, WI, USA).

**MATERIALS AND METHODS**

**Cell Culture**

Ovarian cancer cell line SKOV-3 (ATCC® HTB-77™) was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). SKOV-3 cells were cultured in McCoy’s 5A medium (Thermo Fisher Scientific, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 0.1 mg/ml streptomycin, and 100 U/ml penicillin (Sigma-Aldrich, St. Louis, MO, USA) in 5% CO₂ at 37°C. The CD133+ ovarian CSCs were isolated from SKOV-3 cells using magnetic bead sorting as described previously. Briefly, SKOV-3 cells were dissociated with 0.25% trypsin (Sigma-Aldrich) and resuspended with ice-cold phosphate-buffered saline (PBS). The single cells were incubated with anti-CD133 monoclonal antibody-labeled microbeads (Miltenyi Biotech, Auburn, CA, USA) for 20 min at 4°C in the dark. Then samples were washed with ice-cold PBS and sorted on a BD fluorescence-activated cell sorting (FACS) Aria system (BD Biosciences, San Jose, CA, USA). The sorted CD133+ cells were cultured in a serum-free medium supplemented with 20 ng/ml human recombinant epidermal growth factor and 10 ng/ml human recombinant basic fibroblast growth factor (Invitrogen, Carlsbad, CA, USA).

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In the present study, we constructed two recombinant adenoviruses, Ad-CD133-Cre and Ad-CMV-LoxP-Neo-LoxP-tBid, to achieve and investigate tBid overexpression in CD133+ ovarian CSCs. We hypothesized that Cre/loxP system-mediated tBid overexpression could specifically function in and kill CD133+ ovarian CSCs, thus providing a promising and effective method for preventing the metastasis and recurrence of ovarian cancer.

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RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from sorted CD133+ cells using TRIzol reagent (Invitrogen). The concentration and purity of total RNA were determined using an ultraviolet spectrophotometer (Eppendorf, Hamburg, Germany). The expression of mRNA was analyzed by real-time PCR using primers synthesized by the Shanghai Sangon Biological Engineering and Technology Service (Shanghai, P.R. China). Briefly, 20 µl of reactions containing 50 ng of total RNA, 10 µl of 2× SYBR Green PCR Master Mix, 6.25 µl of AMV reverse transcriptase, 10 µl of RNase inhibitor, and 0.1 mM primers was subjected to one cycle of 95°C for 10 min and then 40 cycles of 95°C for 15 s, 56°C for 30 s, and 72°C for 45 s. The expression of mRNA was normalized to β-actin. Data were analyzed by the ΔCt method and expressed as fold changes.

Western Blot

For cytochrome c detection, cytosolic and mitochondrial proteins were extracted using a mitochondria isolation kit (Pierce, Rockford, IL, USA). For other proteins, the total proteins from the sorted CD133+ cells were extracted using RIPA lysis buffer (Beyotime, Nantong, P.R. China) and quantified with the BCA assay (Pierce). The proteins were loaded and separated by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). After blocking in 2.5% nonfat milk at 37°C for 1 h, the membrane was blotted with primary mouse anti-human antibodies: anti-tBid, anti-cytochrome c, anti-Bax, anti-Bcl-2, anti-caspase 3, anti-caspase 9, and anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. The membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2,000; Bioss Antibodies, Beijing, P.R. China) for 1 h at room temperature. A fluorescent Western blotting detection system was used. The band density of each gene was normalized to the corresponding density of β-actin.

MTT Assay

Cell viability was detected using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, cells were plated onto a 96-well culture plate at a density of 1×10³ cells/well and cultured overnight. Thereafter, cells were infected with Ad-CD133-Cre or/and Ad-CMV-LoxP-Neo-LoxP-tBid and incubated for 48 h. The medium was replaced with fresh medium, and 20 µl of MTT stock solution (5 mg/ml; Sigma-Aldrich) was added to each well. After culturing for 4 h, 200 µl of dimethyl sulfoxide was added to each well to dissolve the formazan crystal. Then absorbance at a wavelength of 490 nm was detected using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA).

Transwell Assay

The invasive behaviors of the indicated cells were analyzed by Transwell chamber (Corning Costar-Corp., Cambridge, MA, USA) assay. In brief, 2×10³ transfected cells in serum-free medium were added to each upper chamber precoated with Matrigel matrix, and 500 µl of McCoy’s 5A medium containing 10% FBS was added to the lower chamber to serve as chemoattractant. After 24 h of incubation, cells on the surface of the upper chamber were removed by scraping with a cotton swab, and the invasive cells on the surface of the lower membrane were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, photographed, and quantified by counting them in five random fields by a light microscope (Olympus, Tokyo, Japan).

Apoptosis Analysis

The apoptotic ratios of cells were determined using Annexin-V–FITC/propidium iodide (PI) apoptosis detection kits (Invitrogen, Shanghai, P.R. China). In brief, cells were first dissociated with trypsin, washed with ice-cold PBS, and resuspended in binding buffer at 1×10² cells/ml. Then the cells were incubated with annexin V and PI stock solution at 4°C in the dark. Thereafter, the samples were detected by FACS, and the data were analyzed by FlowJo software (BD Biosciences).

Animals

Six-week-old female severe combined immunodeficient (SCID) mice, weighing 20–28 g, were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, P.R. China). All experimental animals were housed under specific pathogen-free conditions (21±2°C and 12 h/12 h) with free access to water and food. The animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University Affiliated Suzhou Hospital.

In Vivo Xenograft Experiments

The CD133+ ovarian CSCs (1×10³ cells) were suspended in 200 µl of PBS and injected subcutaneously into the right flank of SCID mice (n=6 mice/group). About 1×10¹⁰ plaque-forming units of recombinant adenovirus diluted in 50 µl of PBS were injected intratumorally every 3 days. Tumor volume was measured using an external caliper and calculated according to the formula: length×width²×π/6. The mice were sacrificed by subcutaneous injection with sodium pentobarbital (100 mg/kg) 30 days after inoculation, and the tumors were excised for Western blot analysis.
Statistical Analysis

All the experiments in this study were performed three times. All data from this study were obtained from at least three independent experiments and were expressed as mean ± SD. All statistical analyses were carried out using the software SPSS version 11.0 for Windows (SPSS Inc., Chicago, IL, USA). Statistical analysis between two samples was performed using Student’s t-test. The chi-square or Fisher’s exact tests were used to evaluate the differences in proportion. Differences were considered significant with a value of \( p < 0.05 \).

RESULTS

Coinfection of Ad-CD133-Cre and Ad-CMV-Loxp-Neo-LoxP-tBid Elevates tBid Expression in CD133+ Ovarian Cancer Stem Cells

To investigate the effect of tBid overexpression in CD133+ ovarian CSCs, the CD133+ cells were sorted from the total SKOV-3 cell line. The purity of CD133+ was 99.97% in sorted cells and 0.94% in total cells, respectively (Fig. 1A). To achieve tBid overexpression in CD133+ ovarian CSCs, we introduced a Cre/loxP-mediated expression system. We first inserted the open reading frame of Cre downstream of the CD133 promoter, which was cloned into an adenovirus (Fig. 1B). Therefore, the Cre would only be expressed when the CD133 promoter was activated. Then we constructed another recombinant adenovirus containing a flanked loxP cassette upstream of tBid. In this way, tBid would only be expressed after deleting the loxP cassette. After a pretest, the CD133+ ovarian CSCs were infected with Ad-CD133-Cre and Ad-CMV-Loxp-Neo-LoxP-tBid at the proportion of 3:2. The transfection efficiency was measured by luciferase activity report after the adenoviruses were marked with green fluorescent protein genes. The results demonstrated that recombinant adenoviruses were successfully transfected into CD133+ cells (Fig. 1C and E). The effect of the Cre/loxP system, the mRNA (Fig. 1F), and protein expression (Fig. 1D and G) of tBid was detected. The results demonstrated that tBid expression levels were significantly increased in the Ad-CD133-Cre and Ad-CMV-Loxp-Neo-LoxP-tBid coinfection group compared with the control group. Additionally, the single infection of

Figure 1. Cotransfection of Ad-CD133-Cre and Ad-CMV-Loxp-Neo-LoxP-tBid elevates tBid expression in CD133+ ovarian cancer stem cells. (A) The percentage of CD133+ in total SKOV-3 cell line and sorted CD133+ were measured by flow cytometry. (B) Diagram of Cre/loxP system-mediated tBid overexpression. The recombinant adenoviruses were infected into CD133+ ovarian cancer stem cells: Control group, CD133+ cells; Empty Ad group, CD133+ cell infected with empty adenovirus; Ad-Cre group, CD133+ cell infected with Ad-CD133-Cre; Ad-Loxp-tBid group, CD133+ cell infected with Ad-CMV-Loxp-Neo-LoxP-tBid; Ad-Cre+Ad-Loxp-tBid group, CD133+ cell coinfected with Ad-CD133-Cre and Ad-CMV-Loxp-Neo-LoxP-tBid. (C, E) The adenoviruses infection efficiency was detected by luciferase activity. (E) The mRNA expression level of tBid was measured by qRT-PCR and normalized to \( \beta \)-actin. (D, G) The protein expression level of tBid was measured by Western blot assay and normalized to \( \beta \)-actin. *** \( p < 0.001 \) versus Control group.
Ad-CD133-Cre or Ad-CMV-Loxp-Neo-LoxP-tBid did not result in tBid overexpression. These results illustrated that Bid overexpression was successfully induced by the recombinant adenovirus Cre/loxP system in CD133+ ovarian CSCs.

**Recombinant Adenovirus Cre/LoxP System-Induced tBid Overexpression Inhibits Cell Proliferation and Invasion of CD133+ Ovarian Cancer Stem Cells**

To evaluate the effect of recombinant adenovirus Cre/loxP system-induced tBid overexpression, the CD133+ ovarian CSCs were coinfected with Ad-CD133-Cre and Ad-CMV-Loxp-Neo-LoxP-tBid. The number of cells was obviously decreased in the coinfected group compared with the control or single recombinant adenovirus infection group (Fig. 2). The cell proliferation rate was measured by the MTT method, and results showed that a single recombinant adenovirus infection had no distinct effect on cell proliferation. Consistent with the former results, coinfection of Ad-CD133-Cre and Ad-CMV-Loxp-Neo-LoxP-tBid significantly inhibited the cell proliferation of CD133+ ovarian CSCs. The invasion ability of CD133+ ovarian CSCs was measured by the Transwell assay. The results demonstrated that cell invasion was significantly decreased in the Ad-CD133-Cre and Ad-CMV-Loxp-Neo-LoxP-tBid coinfected group compared with the other groups.

**Recombinant Adenovirus Cre/LoxP System-Induced tBid Overexpression Increases Cell Apoptosis of CD133+ Ovarian CSCs**

We further investigate the effect of Cre/loxP system-induced tBid overexpression on the apoptotic rate of CD133+ ovarian CSCs. Cell apoptosis was detected by flow cytometric analysis using annexin V-FITC/PI apoptosis detection kits (Fig. 3). The results showed that the coinfecion of Ad-CD133-Cre and Ad-CMV-Loxp-Neo-LoxP-tBid dramatically increased the apoptotic rate in CD133+ ovarian CSCs.

**Recombinant Adenovirus Cre/LoxP System-Induced tBid Overexpression Activates the Mitochondrial Apoptosis Pathway in CD133+ Ovarian Cancer Stem Cells**

It is well known that tBid activated the mitochondrial apoptosis pathway and induced cell death in various cancers. To verify the effect of recombinant adenovirus Cre/loxP system-induced tBid overexpression, we assessed the expression of the mitochondrial apoptosis pathway. The results demonstrated that coinfecion of Ad-CD133-Cre and Ad-CMV-Loxp-Neo-LoxP-tBid significantly upregulated the release of cytochrome c from the mitochondria into the cytosol compared with the other groups. Furthermore, the proapoptosis proteins Bcl-2, caspase 3,
Figure 3. Recombinant adenovirus Cre/loxP system-induced tBid overexpression increases cell apoptosis of CD133+ ovarian cancer stem cells. (A) Cell apoptosis was detected by annexin V-FITC/PI assay. (B) Quantitative statistics of cell apoptosis rate. **p < 0.001 versus Control group.

Figure 4. Recombinant adenovirus Cre/loxP system-induced tBid overexpression activates mitochondrial apoptosis pathway in CD133+ ovarian cancer stem cells. (A) Protein expression level was measured by the Western blot method. (B) Relative protein expression was quantified using Image-Pro Plus 6.0 software and normalized to β-actin. *p < 0.005 versus Control group.
and caspase 9 were significantly increased, while the antiapoptosis protein Bax was obviously decreased in the coinfection group (Fig. 4).

**Recombinant Adenovirus Cre/LoxP System-Induced tBid Overexpression Ameliorates Ovarian Cancer In Vivo**

To investigate the effect of recombinant adenovirus Cre/loxP system-induced tBid overexpression on tumor growth in vivo, the CD133+ ovarian CSCs were coinfected with Ad-CD133-Cre and Ad-CMV-LoxP-Neo-LoxP-tBid and injected into the right scapula of SCID mice. SCID mice injected with blank CD133+ ovarian CSCs were used as the control. The results showed that mice in the coinfection group exhibited a higher survival rate with significantly smaller tumor volume compared with the control group (Fig. 5A–C). Moreover, the tBid expression level was dramatically increased in the coinfection group (Fig. 5D). These results indicated that adenovirus Cre/loxP system-induced tBid overexpression suppressed the CD133-induced cancer growth in vivo.

**DISCUSSION**

Since the discovery of ovarian CSCs, the identification of strategies aimed at eliminating CSCs is of great interest to improve the survival of ovarian cancer.

In the current study, we successfully constructed a recombinant adenovirus Cre/loxP system and achieved conditional tBid overexpression specifically targeting CD133+ ovarian CSCs. Moreover, the tBid overexpression induced by the recombinant adenovirus Cre/loxP system significantly inhibited the tumorigenic potential of CD133+ ovarian CSCs in vitro and in a xenograft model. Taken together, our results reveal a novel adenovirus Cre/loxP system to accomplish tBid overexpression and provide a promising therapeutic strategy in ovarian cancer treatment.

It has been reported that CSCs were associated with tumor initiation, metastasis, chemoresistance, and recurrence. CD133 has been suggested as a stem cell marker.

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**Figure 5.** Recombinant adenovirus Cre/loxP system-induced tBid overexpression ameliorates ovarian cancer in vivo. Control group, SCID mice injected with CD133+ ovarian cancer stem cells; Ad-Cre+Ad-Loxp-tBid group, SCID mice injected with CD133+ ovarian cancer stem cell coinfect with Ad-CD133-Cre and Ad-CMV-LoxP-Neo-LoxP-tBid. (A) The survival percentage of SCID mice. (B) The tumor growth curve of SCID mice. (C) Top image of representative tumors from SCID mice treated with different CD133+ ovarian cancer stem cells. (D) The protein expression of tBid was detected by Western blot and normalized to β-actin. *p<0.005 versus Control group.
for various cancers\textsuperscript{26–29}. Expression of CD133 in cancer-initiating cells is well documented for prostate, brain, breast, and colon cancers, and is indicative of a poor prognosis in many malignant tumors. Ovarian cancer cell lines and primary tumors have been characterized for the expression of several CSC biochemical markers, in which CD133 emerged as the most promising\textsuperscript{30}. In the current study, CD133\textsuperscript{+} CSCs were successfully isolated from the SKOV-3 ovarian cancer cell line, and the purity of CD133\textsuperscript{+} reached 99.97\% in sorted cells. Studies have shown that CD133\textsuperscript{+} ovarian CSCs are more tumorigenic and more resistant to chemotherapeutic treatment\textsuperscript{14}. Thus, CD133\textsuperscript{+} CSCs were an effective and promising therapeutic target for ovarian cancer treatment. However, the method for eliminating CD133\textsuperscript{+} CSCs was limited due to the complex molecular structure of CD133.

In this study, we constructed a Cre/loxP regulation system to selectively augment tBid expression, specifically targeting CD133\textsuperscript{+} ovarian CSCs. It has been reported that the carcinoembryonic antigen (CEA) promoter-mediated Cre/loxP system induces the expression of the cytosine deaminase and specifically inhibits CEA-producing gastric cancer cells in vitro and in vivo\textsuperscript{31}. Similarly, we used a CD133 promoter to control the expression of selective genes. In the current study, we wanted to introduce the suicide gene tBid into CD133\textsuperscript{+} ovarian CSCs and achieve the goal of eliminating CD133\textsuperscript{+} cells. However, it is hard to obtain a recombinant adenosine carrying tBid driven by the CMV potent promoter, due to the cell killing caused by tBid expression during the recombinant adenosine production. Thus, we needed to construct a conditional tBid expression system. Therefore, the Cre/loxP regulation system was used to elevate the selective expression of tBid. Cre is a site-specific recombinase that can mediate the specific recombinant process between two loxP sequences\textsuperscript{32,33}. We first constructed Ad-CMV-LoxP-Neo-LoxP-tBid, a recombinant adenosine carrying tBid (with CMV promoter), and a null gene expression cassette (Neo) flanked by loxP was inserted upstream of tBid to block tBid expression. To conditionally initiate tBid expression in CD133\textsuperscript{+} stem cells, we constructed another recombinant adenosine, Ad-CD133-Cre, carrying Cre with a CD133 promoter inserted upstream of Cre. In this way, the infection of Ad-CD133-Cre into CD133\textsuperscript{+} ovarian CSCs would trigger the expression of Cre, which would then mediate the loxP recombinant and cut off the null gene expression cassette between the two loxP, ultimately leading to tBid overexpression\textsuperscript{34}. In our study, coinfection of Ad-CMV-LoxP-Neo-LoxP-tBid and Ad-CD133-Cre significantly elevated the expression of tBid, suggesting that we successfully introduced tBid into CD133\textsuperscript{+} ovarian CSCs.

tBid is normally reported to induce cell apoptosis in various cancer both in vivo and in vitro\textsuperscript{35}. The results in our study demonstrated that tBid overexpression was induced by the recombinant adenosine Cre/loxP system, and it not only significantly increased cell apoptosis but also inhibited cell proliferation and invasion. This suggested that Cre/loxP system-mediated tBid overexpression suppressed the tumorigenic potential of CD133\textsuperscript{+} ovarian CSCs. Moreover, the coinfection of Ad-CMV-LoxP-Neo-LoxP-tBid and Ad-CD133-Cre dramatically increased the mitochondrial release of cytochrome c; promoted the expression of proapoptotic protein Bax, caspase 3, and caspase 9; and inhibited the antiapoptotic protein Bcl-2. These results suggested that Cre/loxP system-mediated tBid overexpression activated the mitochondrial apoptosis pathway in CD133\textsuperscript{+} ovarian CSCs. Additionally, we verified the effect of adenosine Cre/loxP system-mediated tBid overexpression in a xenograft model. The results were consistent with the in vivo study, further identifying that Cre/loxP system-mediated tBid overexpression increased the survival of CD133\textsuperscript{+}-induced ovarian cancer.

In summary, the present study established and applied the adenosine Cre/loxP system to introduce selective tBid expression in CD133\textsuperscript{+} ovarian CSCs. The overexpression induced by the adenosine Cre/loxP system significantly inhibited the tumorigenic potential of CD133\textsuperscript{+} ovarian CSCs both in vivo and in the xenograft model, providing a new therapeutic approach for ovarian cancer treatment.

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