

Original Article

Silencing Bmi1 inhibits the proliferation and invasiveness in CD133⁺ cancer stem cells of Hep-2 cells

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Abstract: It has been shown that over-expression of B-cell-specific Moloney murine leukemia virus insertion site 1 (Bmi1) play a key role in maintaining the stem-like properties in CD133⁺ Hep-2 cells. This study was aimed at investigating the effects of silencing Bmi1 on CD133⁺ Hep-2 cells. Bmi1 silencing was achieved by transfection with the vectors expressing small hairpin RNA (shRNA) versus Bmi1. Cell proliferation was detected in a micro plate reader with Cell Counting Kit-8, cell apoptosis was analyzed by flow cytometry and Cell invasion were detected by trans-well invasion assays. The study found that silencing Bmi1 inhibited the proliferation of Hep-2 CD133⁺ cells, and that the proportion of apoptotic cells were enhanced and invasion were inhibited by knockdown of Bmi1 in CD133⁺ Hep-2 cells. Furthermore, mRNA and protein of P16 were increased strikingly after Bmi1 silencing. The over-expression of Bmi1 is responsible for stem-like properties in CD133⁺ Hep-2 cells, indicating that silencing Bmi1 expression in the cells might be developed as an efficient therapy.

Keywords: Bmi1, shRNA, CD133⁺ cells, cell proliferation, apoptosis, invasion

Introduction

In the northeast area of China, laryngeal carcinoma is one of the most common malignancies which are difficult to cure. Despite the improvement of surgery and the combination with radio- and chemotherapy, the overall survival has not improved substantially. Accumulating evidence suggest that cancer stem cells (CSCs) may play a pivotal role in the failure to respond to treatment or tumor relapse [1, 2]. CD133 is a putative marker for cancer stem cells in laryngeal carcinoma as well as in many other types of solid tumor [3, 4]. In addition to the higher clonogenicity, invasiveness and tumorigenesis properties, CD133⁺ cancer stem cells have also been shown to establish more efficiently in immunocompromised mice and display a high resistance to conventional chemotherapy [5, 6].

B-cell-specific Moloney murine leukemia virus insertion site 1 (Bmi1) is a member of the poly comb family of chromatin-modifier proteins. It has been shown to depress the expression of the Ink4a locus which encodes for two tumor

suppressor proteins p16Ink4a and p14Arf, thereby promoting cell proliferation and postponing senescence [7]. Then, Bmi1 has been shown to play a role in tumorigenesis in Ink4A deficient models, suggesting that it may regulate other genes important for cancer [8]. Bmi1 regulates self-renewal in both normal and cancer stem cells, including HNSCC [9-13]. Bmi1 overexpression has been frequently observed in a series of human cancers with diverse functional roles [14-16]. Bmi1 knockdown enhanced the sensitivity of breast cancer cells to 5-Fu [17], the Bmi1 gene was overexpressed in 5Fu-resistant cells, and Bmi1 knockdown effectively reversed chemoresistance [18].

Recently, we and other groups have identified the CD133⁺ CSCs in Hep-2 cells [6, 19]; our previous study showed that Bmi-1 was highly expressed in these CD133⁺ cells [20]. However, while the genes and pathways associated with over-expressed Bmi1 have been frequently reported, little is known about the proliferation and invasiveness after the high level expression of Bmi1 is knocked down in CSCs.

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Table 1. Primers for Bmi1-shRNA

	Primers	Sequences	Position
Bmi1-shRNA1	Forward	5'-AAAGTTTCAACAAGACCAGACCAC-3'	176
	Reverse	5'-CAAGTGTCTGGTCTGGTAAAA-3'	
Bmi1-shRNA2	Forward	5'-AAAGGATACTTACGATGCCAGC-3'	483
	Reverse	5'-CTATGAATGCTACGGGTCGAAAA-3'	
Bmi1-shRNA3	Forward	5'-AAAGGATCAGTCACCAGAGAGAT-3'	703
	Reverse	5'-CTAGTCAGTGGTCTCTCTAAAAA-3'	

The present study was carried out to seek the role of Bmi1 in CD133⁺ cancer stem cells and whether the knock-down of Bmi1 suppress the feature of CD133⁺ cancer stem cells. The effect of silencing Bmi1 on cell proliferation, apoptosis, invasion and the expression of related genes were analyzed.

Materials and methods

Materials

Dulbecco's Modified Eagle Medium (DMEM) was from Gibco BRL (Carlsbad, CA, USA). Fetal bovine serum (FBS) was from HyClone Inc. (Logan, UT, USA). Trizol Reagent, PCR primers, and RT-reaction Kit were purchased from TaKaRa Biotechnology (Dalian, Liaoning, China). LipofectamineTM2000 were from Invitrogen (Carlsbad, CA, USA). Cell Counting Kit-8 (CCK-8) was from Dojindo (Tokyo, Japan). Cell Invasion Detection Kit includes chamber of 24-well cell culture inserts with 8 μm pores (Corning, Medfield, Massachusetts, USA) coated with Matrigel (Invitrogen, Carlsbad, USA).

Construction of shRNA expression vector silencing Bmi1 and cell culture

Three oligonucleotides as DNA template encoding shRNA-Bmi1, cloning shRNA template into double-promoter pFIV-H1/U6 shRNA vector were designed and synthesized. The primer sets are shown in **Table 1**. A scramble oligonucleotide which has noninterference to any human mRNA was chosen as control-shRNA.

The cell line Hep-2 was kindly provided by the central laboratory of the Second Clinical Hospital of Jilin University. These cells were cultured in DMEM supplemented with 10% fetal calf serum 100 IU/ml penicillin, 100 μg/ml streptomycin. Applying the fluorescence-activated cell sorting (FACS) technology as we previously described [6], CD133⁺ cancer stem

cells of hep-2 cells were sorted. All the cultures were maintained at 37°C in a humidified 5% CO₂ incubator.

Transfection of recombinant plasmid

Transfection was performed using LipofectAMINE 2000 reagent (Invitrogen Life Technologies),

according to the manufacturer's instructions. After 72 h, Cells underwent lysis for western blot assay, and total RNA was extracted using Trizol reagent. Then the plasmid sequence that had the best knockdown effect was chosen from the three plasmids according to the mRNA and protein level of Bmi1. CD133⁺ Cells were transfected with the best plasmid and following experiments were performed.

RT-PCR analysis and western blot analysis

The different group cells including 3 Bmi1-shRNA, control-shRNA and blank control CD133⁺ cells were harvested. Total RNA purification was performed using RNA Isolation Kit (Promega, USA) according to the manufacturer's protocol. The Bmi1 mRNA expression by RT-PCR analysis and protein expression by Western blot analysis were performed as we previously described [20].

The following P16 mRNA expression by RT-PCR analysis and protein expression by Western blot analysis were performed as the same method with the following primers: P16, Sense 5'-AGGCGAACTCGAGGAGAGC-3', Antisense 5'-GTACGACCGAAAGTGTTCG-3'.

Cell proliferation assay

Cell viability was evaluated using the Cell Counting Kit-8 (CCK-8) (Dojindo, Japan) according to the manufacturer's instructions. Briefly, the cells were incubated with CCK8 solution and then the absorbance was determined at 450 nm wavelength in a micro plate reader. The absorbance values were converted to cell numbers from a standard curve of cell numbers against absorbance value which resulted from a series of analyses of samples in which the cell numbers were known. The tests were repeated for three times, and the mean value was calculated.

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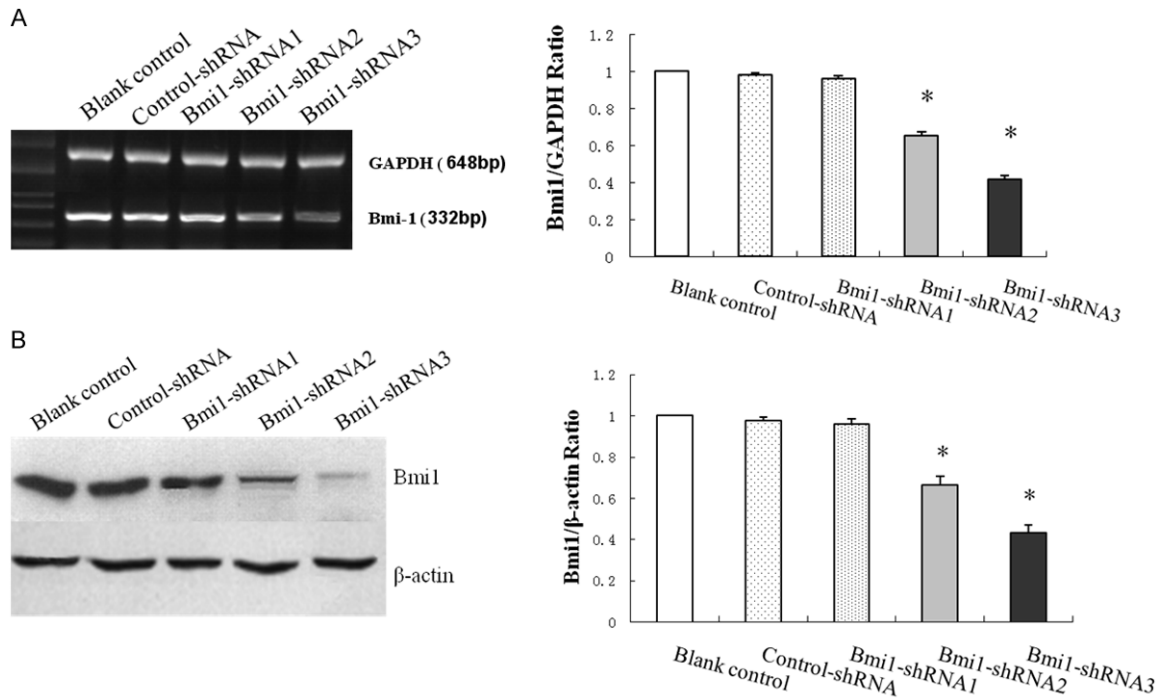


Figure 1. Expression of Bmi1 in CD133⁺ hep-2 cells transfected with various plasmids. A. RT-PCR analysis of Bmi1 mRNA. B. Western blot analysis of Bmi1 protein. **P* < 0.05, versus the two control groups.

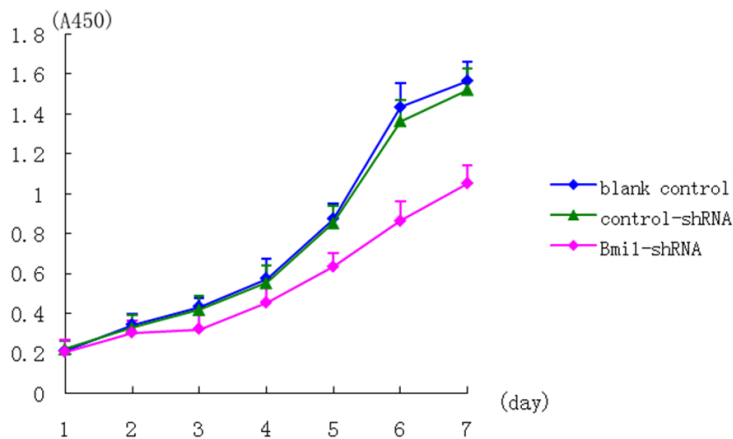


Figure 2. Silencing Bmi1 inhibited the proliferation of CD133⁺ cells. The CCK-8 assay were applied to examine the relative proliferation rate of different group cells, silencing Bmi1 inhibited the proliferation of CD133⁺ cells.

Detection of apoptosis by flow cytometry

The sorted CD133⁺ cells were transfected with Bmi1-shRNA and control-shRNA, respectively. After 48 h, these cells and control group CD133⁺ cells were harvested. After treatment with 70% ethanol for more than 12 h, cells were washed with PBS twice. Then, cell density was adjusted to 1×10^6 cells/ml. Following RNAase

digestion, cells were incubated in 1.5 ml of propidium iodide (PI) at 5 mg/100 ml for 30 min. Then, flow cytometry was performed. A total of 1×10^4 cells were detected for each sample.

Cell invasion assay

Cells were seeded at a density of 5×10^4 per well in the upper inserts with 8 μ m pores in serum-free DMEM coated with Matrigel and the lower chamber were filled with DMEM containing 10% FBS. Cells were incubated at 37°C with 5% CO₂ for 48 h. The cells that invaded across the membrane were stained with HE stain and were

photographed under a microscope using a 10 \times objective. The invaded cells were counted manually in random 5 fields per well and characterized as mean \pm standard deviation (SD).

Statistical analysis

We used SPSS for Windows version 13.0. Data are expressed as mean \pm SD and evaluated

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Table 2. Percentage of apoptosis cells 48 hours after transfection with shRNA Bmi1

Group	Apoptotic cells (%)
Blank control	0.74 ± 0.18
Negative contro	1.13 ± 0.35
Bmi1-silenced group	9.5 ± 1.25*

*P < 0.05 vs blank control and negative control.

with independent-sample *t*-tests. A value of *P* < 0.05 was considered statistically significant.

Results

Expression of Bmi1 was down-regulated by the shRNA expression vector

In our previous study, CD133⁺ cells were isolated from the Hep-2 cell culture by FACS sorting. The Bmi1 levels of expression were higher in the CD133⁺ cells, suggesting that Bmi1 might play a crucial role in maintaining the stem-like properties in these cells [20]. To silence the high expression of Bmi1 and study its roles in CD133⁺ hep-2 cells, three Bmi1-shRNA plasmids were constructed and transfected into CD133⁺ cells. As shown in **Figure 1**, the expression of both Bmi1 mRNA and protein was successfully silenced. The mRNA and protein expression of Bmi1 was significantly reduced in the CD133⁺ cells transfected with Bmi1-shRNA2 and Bmi1-shRNA3 when compared with control-shRNA and blank control group, (*P* < 0.01). The mRNA inhibition rates were 65.4% and 41.5%, the protein inhibition rate was 66.4% and 43.4%. In the following experiments, we selected the Bmi1-shRNA3 plasmid which has the best knockdown effect.

Silencing Bmi1 inhibited the proliferation of CD133⁺ cells

The proliferation of CD133⁺ cells was analyzed to explore the effects of silencing Bmi1 in the cells. As shown in the **Figure 2**, silencing Bmi1 could significantly inhibit the proliferation of CD133⁺ cells. After 5 days, the growth of CD133⁺ cells (Bmi1-shRNA) in which Bmi1 had been silenced was significantly inhibited when compared with the blank control and transfection control (control-shRNA) cells (*P* < 0.01).

Bmi1 silencing enhanced the apoptosis of CD133⁺ cells

Flow cytometry showed, 48 h after transfection with Bmi1-shRNA, the apoptotic CD133⁺ cells

were significantly increased to 9.5% ± 1.25%. No or few apoptotic cells were found in the negative control-shRNA group and the blank control group. In addition, the apoptotic cells significantly increased in Bmi1-shRNA group as compared to the control-shRNA group (*P* < 0.05) (**Table 2**); while there was no significant difference between control-shRNA group and blank control group (*P* > 0.05), indicating no influence of negative control plasmid on the apoptosis of CD133⁺ cells.

Bmi1 silencing suppressed the invasiveness of CD133⁺ cells

Figure 3 shows a representative photograph of the cell invasion and the statistical analysis of the effects of silencing Bmi1 on cell invasion. Numbers of invasive cells in the Bmi1-shRNA group (64 ± 9.6) was lower than that of control-shRNA group (81 ± 8.2) and the blank control group (88 ± 9.7) in vitro (*P* < 0.05). While no significant difference of the invasiveness was found between control-shRNA group and the blank control (*P* > 0.05). The data indicate that silencing Bmi1 decreased the cell invasion, suggesting that high expression of Bmi1 in CD133⁺ cells contributes to the elevated cell invasion.

Bmi1 silencing changed the expression of related gene P16

Based on the above findings and the fact that Bmi1 regulate the expression of the Ink4a locus which encodes for two tumor suppressor proteins p16Ink4a and p14Arf, thereby regulating cell proliferation and senescence [7]. We used RT-PCR and western blot to analyze the expression of mRNAs and protein of P16 in order to examine the possibility that Bmi1 might exert its role through the regulation of gene expression. As shown in the **Figure 4**, the mRNA and protein expression of P16 was significantly increased in Bmi1-shRNA when compared with control-shRNA and blank control group, (*P* < 0.01) indicating that the silencing of Bmi1 increases the P16 expression in CD133⁺ cells.

Discussion

There is increasing evidence that cancers contain a small subset of their own stem like cells called CSCs [21, 22]. Cancer stem cell hypothesis suggests that only CSCs have the exclusive self-renewal capacity to form new tumors [23,

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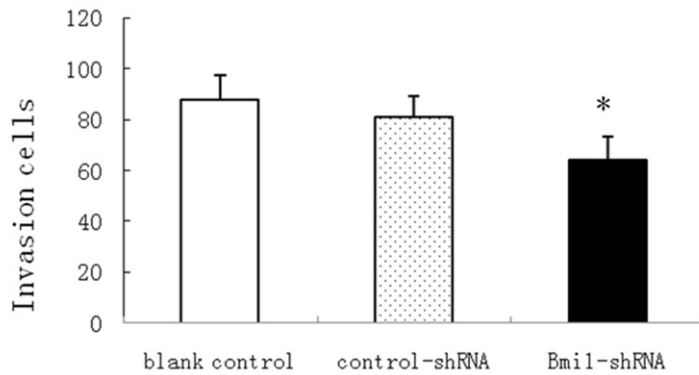
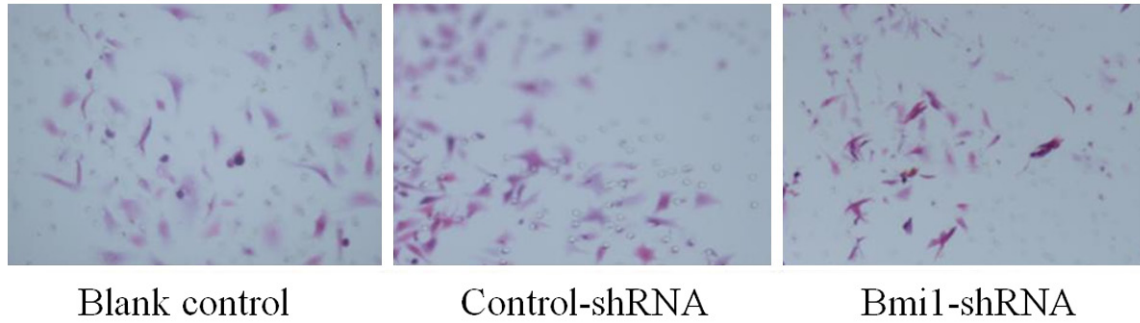


Figure 3. Bmi1 silencing decreased cell invasion, the cell invasion assay and statistical analysis were carried out as described in the Materials and Methods. The cells passed through the membranes were showed by microscopic photographs and statistical analysis showing that the number of cells passing through the membranes. The data are expressed as the mean ± SD, *P < 0.05, versus blank control and control-shRNA cells.

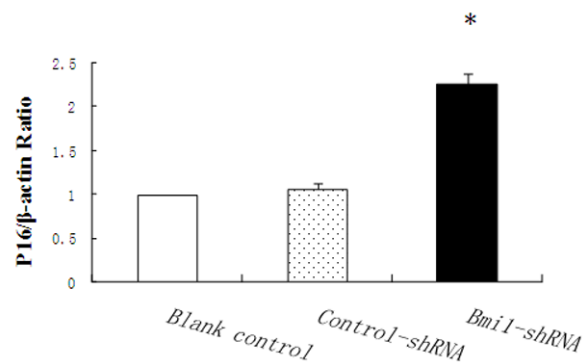
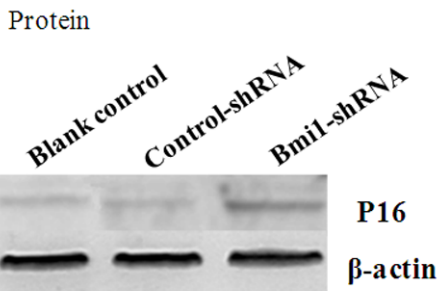
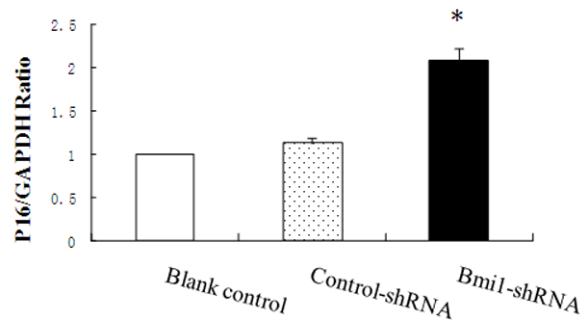
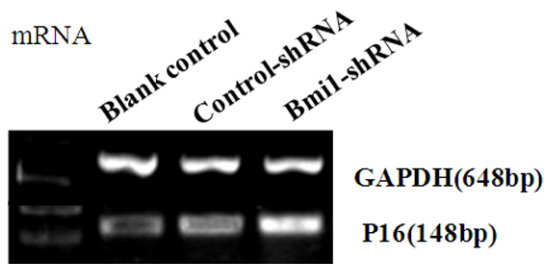


Figure 4. Expression of P16 was enhanced by silencing Bmi1 in CD133⁺ cells. The figure shows that silencing of Bmi1 lead to increases of P16 mRNA and protein. The data are expressed as mean ± SD, *P < 0.05 black control and control-shRNA control cells.

24]. Although it is still not clear whether the cancer stem cells are derived from original tis-

sue-derived stem cells, bone marrow stem cells, or mature cells that have undergone a

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dedifferentiation process, it has been suggested that novel strategies for successful cancer therapy should focus on the elimination of cancer stem cells [25].

Bmi-1 was first identified in 1991 as a frequent target of Moloney virus insertion in virally accelerated B-lymphoid tumors of E mu-myc transgenic mice [26]. Since then, Bmi1 has been associated with several cancers including non-small cell lung cancer, ovarian cancer, acute myeloid leukemia, nasopharyngeal carcinoma, glioblastoma, and breast cancer [27]. Bmi1 is critical to invasive potential and contributes to the maintenance and self-renewal of CSCs [9, 28, 29]. Chou found that Bmi1 overexpression drives stem-like properties associated with invasion, metastasis, and poor prognosis in HNSCC [10]. RNA interference is a powerful tool to study gene function and evolutionarily conserved process that functions to inhibit gene expression [30, 31]. Notably, a number of studies have explored the use of shRNA to silence the expression of oncogenes and specific targets that promote the proliferation of tumor cells as an anticancer strategy [32]. T Randall et al have developed a new vector with opposing convergent promoters named "HI inverted U6 promoter plasmid" for the expression of shRNAs, which can be used to knock down endogenous genes in a high throughput manner [33]. Short double-stranded oligonucleotides can be easily and efficiently introduced into this vector to knock down any mRNA, or shRNA expression cassettes can be generated in a high throughput manner by PCR.

In our previous studies we sorted CD133⁺ cancer stem cells using flow cytometry from the Hep-2 cell line and found that Bmi-1 was highly expressed in these CD133⁺ cells. Bmi-1 may play a key role in the stem feature of CD133⁺ cancer stem cells in hep-2 cells [6, 20]. So we designed three pFIV-H1/U6 shRNA plasmids to knock down Bmi1 gene. Our present results showed that pFIV-H1/U6 Bmi1-shRNA could be transfected into CD133⁺ cells and silencing of Bmi1 (pFIV-H1/U6 shRNA) broadly changed the expression of Bmi1 significantly, as compared to the expression in the control cells. The best silence effect plasmid was selected to do the following studies.

In this study, Cell proliferation was detected with cell counting kit8 and cell apoptosis was analyzed by flow cytometry. CCK-8 assay show-

ed that with Silencing of Bmi1 the growth of CD133⁺ cells was inhibited and flow cytometry analysis showed that with Silencing of Bmi1 the apoptotic cells were markedly increased. The apoptosis rate was $9.5 \pm 1.25\%$ in the Bmi1-shRNA group, which is significantly elevated when compared with the control-shRNA group and the blank control group. The results indicate that Bmi1-shRNA is effective to inhibit CD133⁺ cell proliferation and enhanced CD133⁺ cell apoptosis.

Invasion is a prerequisite for metastasis and is usually positively correlated with the metastatic potential of malignant tumor cells [34]. Bmi1 has been reported to be responsible for the invasion of some epithelial derived tumor cells. For example, High level of Bmi1 is associated with invasion and is also considered to be a poor prognostic marker in multiple human cancers [35], and is significantly involved in chemoresistance and tumor recurrence [17, 36]. Silencing Bmi1 gene with lentivirus-mediated shRNA led to elimination of the tumor-forming capacity, particularly of CD133⁺ tumor cells, thereby supporting the development of new targeted therapies against Bmi1 in pGBM [37]. The present study shows that silencing Bmi1 significantly suppresses the invasive ability of CD133⁺ cancer stem cells, indicating that abnormal expression of Bmi1 is responsible for the elevated invasion of the cells, suggesting its potential role in the metastasis.

More and more evidences have been accumulated to show that the exogenous P16 (INK4a) or p14 (ARF) can inhibit the cell proliferation and/or induce the apoptosis [38]. P16 also has been reported to rise and inhibit CDK activity only after human fibroblasts have completely arrested due to replicative senescence [39]. The expressions of the related gene P16 was significantly enhanced as compared to the expression in the control cells.

We also plan to conduct experiments to examine the effects of silencing Bmi1 on the resistance of CD133⁺ cells to chemotherapeutic agents used to treat laryngeal carcinoma. And understanding the molecular mechanisms through which silencing Bmi1 led to tumor abrogation is important for future development of targeted therapies.

In this study we found that the proliferation and invasion were suppressed and the apoptosis

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were enhanced by silencing Bmi1 in CD133⁺ cancer stem cells of hep-2 cells. These results indicate that the over-expression of Bmi1 in CD133⁺ cancer stem cells contributes to maintenance of the malignant characteristics and suggest that silencing Bmi1 in these cells might improve the therapeutic effects in laryngeal carcinoma.

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Disclosure of conflict of interest

None.

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