BMI1 promotes the progression of laryngeal squamous cell carcinoma

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ABSTRACT

BMI1 is highly expressed in several malignant tumors, and its expression level is associated with tumor progression, proliferation, and prognosis. However, no published studies have examined the role of BMI1 in laryngeal squamous cell carcinoma (SCC).

Expression of BMI1 in primary tumors was analyzed by immunofluorescence staining, real-time PCR, and Western blotting. BMI1 was knocked down, and proliferation, apoptosis, and cell cycle assays were performed. Sensitivity to radiochemotherapy was evaluated, and tumorigenicity assays were performed in vivo.

BMI1 was highly expressed in laryngeal SCCs. BMI1 promoted cell proliferation and tumor progression, and inhibited apoptosis due to influences on the cell cycle. More importantly, BMI1 suppressed the sensitization of laryngeal Hep2 cells to radiochemotherapy.

BMI1 is essential to maintain the proliferation and progression of laryngeal SCCs. Therefore, depletion of BMI1 may be a potential therapeutic option for cancer management.

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INTRODUCTION

Laryngeal squamous cell carcinoma (SCC) is one of the most common malignancies in the head and neck. Despite major improvements in the treatment of laryngeal carcinoma, including surgical techniques and chemoradiotherapies, some cases of SCC remain incurable. With the rapid development of molecular biological techniques, gene therapy is attracting more attention and is regarded as the hope of cancer patients.

B-cell-specific Moloney murine leukemia virus insertion site 1 (BMI1) is an important component of the polycomb repressive complex 1, which is a key epigenetic regulator. Through chromatin and histone modifications like methylation, BMI1 controls the cell cycle and self-renewal of tissue stem cells.1 BMI1 contains a ring finger domain at its N terminal, in the center of which there is a helix-turn-helix-turn motif (H-T-H-T). Through this ring finger structure, BMI1 plays key roles in cell proliferation and repression of protein expression.2 The ring finger of BMI1 is a special type of zinc finger that links two zinc atoms and is involved in viral replication and signal transduction. BMI1 cooperates with c-Myc protein to suppress the Ink4a/Arf locus, negatively regulate the transcription of p16ink4a and p19arf, and inhibit cellular aging.3 It has been reported that BMI1 determines the proliferation of hematopoietic stem cells,3 neural stem cells,4 and mammary stem cells,5 and also sustains the proliferation capacity of leukemic stem cells6 and glioblastoma stem cells.7 In particular, BMI1 is highly expressed in multiple malignant tumors, including breast cancer,8 colorectal cancer,9 prostate cancer,10 metastatic melanoma,11 nasopharyngeal carcinoma,12 non-small cell lung cancer,13 and oral squamous cell carcinoma.14 These observations led us to hypothesis that BMI1 functions in other human cancers.

However, the BMI1 gene has not been investigated in human laryngeal cancer. In this study, we were the first to identify the significant expression of BMI1 in primary laryngeal SCCs, and through shRNA-mediated silencing, we investigated whether BMI1 affects the proliferation and progression of laryngeal cancer in vitro and in vivo.

MATERIALS AND METHODS

Primary laryngeal SCC specimens

Twenty primary laryngeal SCC specimens and 20 paracancerous epithelial tissue samples were obtained from consenting patients. All procedures were approved by the Ethics Boards at the Affiliated Eye and ENT Hospital of Fudan University (Shanghai, China).

Cell culture

The Hep-2 cell line was established in 1952 from tumors produced in irradiated cortisonized weanling rats after injection with epidermoid carcinoma tissue from the larynx of a 56-year-old woman.
old male. His carcinoma tissue had an epithelial-like morphology and was resistant to temperature, nutritional, and environmental changes without loss of viability. The Hep-2 cell line was cultured in RPMI-1640 medium (GIBCO, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, GIBCO) in a humidified incubator at 37 °C with 5% CO₂. Cells plated at a concentration of 1 × 10⁶ cells/cm² grew to 100% confluence in 3 days.

**Immunofluorescence staining**

Tissue samples were fixed in 4% paraformaldehyde at room temperature for 24 h, and then tissue blocks were embedded in paraffin, cut to the desired thickness of 5 µm, and affixed onto the slide. Before proceeding with the staining protocol, the slides were deparaffinized and rehydrated. Heat-induced epitope retrieval was performed to break the methylene bridges and expose the antigenic sites. Slides were washed and incubated overnight at 4 °C with primary antibody. After washing three times in phosphate-buffered saline (PBS), fluorophore-conjugated secondary antibody was applied to the slide and incubated for 1 h at room temperature. Slides were mounted on coverslips in DAPI-containing mounting medium (Sigma, St. Louis, MO, USA). Slides were examined under a fluorescence microscope (Leica DMi8B, Wetzlar, Germany), and images were captured with a digital camera (Leica QWin software). The primary antibody was mouse anti-human BMI1 (Abcam, Cambridge, MA, USA) and the secondary antibody was CY3-conjugated goat anti-mouse (Sigma).

**Real-time polymerase chain reaction (PCR)**

Total RNA was isolated from fresh primary samples using TRIzol reagent (Invitrogen). Reverse transcription was performed with 1 µg total RNA and MMLV reverse transcriptase (Promega, Madison, WI, USA). PCR amplification was performed using Taq polymerase (Sangon Biotech, Shanghai, China) under the following conditions: denaturation at 94 °C for 5 min, and then 40 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 58 °C, and extension for 20 s at 72 °C. Real-time PCR was performed using the Syber green PCR Master Mix (Takara Biotech, Dalian, China) and a real-time PCR apparatus (iCycler iQ5, Bio-Rad, Hercules, CA, USA). The BMI1 gene was amplified using the primers 5'-ATGATAAAAGATACTTACGATGCCCAG-3' (forward) and 5'-GAACCTGTTATTTACATGGAAGTCGAC-3' (reverse). GAPDH was used as an internal marker and was amplified using the primers 5'-AGAGGCTGGGGCTCATTTG-3' (forward) and 5'-AGGGGCCATC-CACAGTCTTC-3' (reverse). For calculation of differential gene expression, the 2⁻ΔΔCt formula was used.

**Western blotting**

Fresh primary samples were processed with cell lysis reagents (Cell Signaling Technology, Danvers, MA, USA). Twenty micrograms of protein per sample was loaded and separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), followed by electrophoretic transfer onto a nitrocellulose membrane (Millipore, Bedford, MA, USA) using the Bio-Rad transfer system. After blocking with 30 ml Tris-buffered saline containing 0.05% Tween-20, the membrane was first incubated with mouse anti-human BMI1 (Abcam) and mouse anti-human β-actin (Sigma) antibodies. Membranes were treated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and developed using the ECL substrate (Thermo Fisher Scientific, Waltham, MA, USA). The relative amount of protein on the blots was determined by densitometry using LabWorks software (UVP, Upland, CA, USA). β-actin was used as a loading control. The relative expression of BMI1 = sample gray value/actin gray value.

**Construction of short-hairpin RNA and lentiviral infection**

BMI-1 small hairpin RNA (shRNA) was designed to target the BMI1 opening reading frame (ORF) as previously reported.7 The upstream shRNA sequences were as follows: Oligo1, CCTAATCTTTC-CAGATTGAT, and Oligo2, TACAATCTGGAAAGTATTAGG. The downstream shRNA sequences were oligo3, CCTAATCTTCCAT-ATTGAT, and oligo4, TACAATCTGGAAAGTATTAGG. The interfering plasmids, plasmids pšsv-REV, pMDig-pRRE, and pMD2.G, which contain the lentiviral vector system Tronolab, were transfected into 293T packing cells using the calcium phosphate precipitation method. The cellular supernatant containing lentivirus was collected, filtered, and concentrated by ultracentrifugation. Lentiviral titers were measured by flow cytometry after infection of 293T cells. Lentiviruses containing BMI1 shRNA were transfected into the laryngeal Hep-2 cell line. Transfection efficiency was evaluated by quantitative real-time PCR.

**Proliferation assay**

Cell proliferation capacity was evaluated using the Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer’s instructions. Cultured Hep2 cells and RNA-interference cells were plated in 96-well microwell plates in 0.1 ml RPMI 1640 with 10% FBS at a density of 2 × 10³ cells/well. Each group of cells were plated in six microwells, and medium was changed every 3 days. Proliferation assays were performed on days 1, 3, 5, and 7 after RNA interference. Ten microliters of CCK-8 dye was added to the wells and incubated for 2 h. The ultraviolet absorbance at 450 nm was measured using an ELISA microplate reader (Biorad 680, Bio-Rad).

** Colony formation assay**

Cultured Hep2 and RNA-interference cells were trypsinized and pipetted into single cell suspensions with RPMI 1640 supplemented with 10% FBS. The cells were transferred into 96-well plates using the limited dilution method such that only one cell was allowed in each well for growth. After 2 weeks, cultured colonies were stained with Toluidine blue (Sinopharm Chemical Reagent Co., Shanghai, China). To evaluate the colony formation quantitatively, a fixed measuring frame were used and calculate the percentage of the areas of the colonies. The percentage of colony area = (area of colony/area of measuring frame) × 100.

** Apoptosis assay**

An apoptosis assay was performed to evaluate apoptosis in both Hep-2 cells and RNA-interference cells. Cells were suspended in 200 µl PBS at 2 × 10⁷ cells/ml and stained with 50 µl TUNEL reaction mixture (In Situ Cell Death Detection Kit, Fluorescein, Roche, South San Francisco, CA, USA). DAPI-containing mounting medium (Sigma) was used to counterstain the nuclei and the samples were observed under a fluorescence microscope (Leica DMi8B). To confirm the morphological TUNEL results quantitatively, a flow cytometric method was also used. Cultured Hep-2 cells and RNA-interference cells were incubated with RNase A (Takara Bio, Otsu, Japan) for 30 min and stained with 50 µg/ml propidium iodide (PI, Sigma) in the dark at 4 °C for 60 min. The cells were analyzed by flow cytometry (Beckman Coulter, Brea, CA, USA), and the apoptotic rate was calculated. The necrotic cells could be differentiated with forward scatter and side scatter gating because the forward...
scatter and side scatter of necrotic cells were both obviously increased. For better performance of this experiment, the growth cycle and morphological homogeneity of the cells were carefully maintained to ensure that the cells were in good condition and to decrease the possibility of necrosis.

Cell cycle assay

The cell cycle was analyzed using flow cytometry with PI staining. Hep-2 and RNA-interference cells (1 × 10⁴ cells) were washed with PBS and fixed overnight in ice-cold 70% ethanol. The cells were washed twice with PBS, treated with 10 mg/ml RNase A for 30 min, and stained with 50 μg/ml PI in the dark for 1 h. The cell cycle was analyzed by flow cytometry (Beckman Coulter), and the PI fluorescence was measured at 488 nm. Each sample of 1 × 10⁴ cells was analyzed in triplicate.

Radiotherapy sensitivity assay

Hep-2 and RNA-interference cells in RPMI 1640 with 10% FBS were each plated in 12 wells of a 96-microwell plate at 1 × 10⁴ cells/well. In each plate, six wells were irradiated and the other six wells were not exposed to irradiation and considered as controls. The doses of irradiation were 2, 4, and 8 Gy. After 48 h, the cell survival rate (SR) was calculated by the CCK-8 method. Ten microliters of CCK-8 dye was added to all wells and incubated for 2 h. The ultraviolet absorbance at 450 nm was measured using an ELISA microplate reader (Biorad 680).

SR = (mean absorbance of the test well/mean absorbance of the control) × 100%; inhibition rate (IR) = 100% − SR.

Chemotherapy sensitivity assay

Hep-2 and RNA-interference cells in RPMI 1640 with 10% FBS were each plated in 12 wells of 96-microwell plates at 1 × 10⁴ cells/well. In each plate, six wells of each group of cells were treated with Cisplatin Injection (Nanjing Pharmaceutical Factory Co., Nanjing, China), and the other six wells were not treated. The doses of cisplatin were 3, 6, and 12 μg/ml. After 24 h, the SR and IR were determined by the CCK-8 method as above. All experiments were performed three times.

In vivo tumorigenicity assay

NOD-SCID mice were obtained from Shanghai Slac Laboratory Animals (Shanghai, China). For observation of tumor growth in vivo, Hep-2 and RNA-interference cells (1 × 10⁵ cells/mouse) were injected into the subcutaneous space of the axillary fossa of mice under anesthesia with ketamine/xylazine. The mice were allowed to grow for 8 weeks and were examined every 3 days for tumor growth by palpation. At the end of 8 weeks, all mice were sacrificed by cervical dislocation, and tumors were isolated, weighed, and fixed in paraformaldehyde. Each tumor nodule was confirmed by hematoxylin-eosin staining.

Statistical analysis

All the results are expressed as mean ± standard deviation (SD) for at least three independent experiments. Statistical differences were analyzed by independent-sample t-tests. Stata 7.0 (StataCorp LP, College Station, TX, USA) and Microsoft Office Excel 2003 were used for data processing and statistical comparisons between Hep-2 and RNA-interference cells. Values of p < 0.05 were considered statistically significant.

Results

BMI1 was highly expressed in laryngeal carcinomas

Since BMI1 was reported to be associated with multiple cancers but has never been investigated in laryngeal carcinoma, we decided to determine whether BMI1 is highly expressed in primary laryngeal carcinomas. The primary tumors in the experiments were graded from moderately to poorly differentiated. Using immunofluorescent staining, we found that BMI1 was frequently and clearly expressed in laryngeal tumors (Fig. 1A). To determine the expression of BMI1 quantitatively, real-time PCR was performed on both primary tumor samples and paracancerous tissues. The results revealed that the expression of BMI1 in tumor samples was much higher than in paracancerous tissues (p < 0.05) (Fig. 1Bi). To understand the relationship with clinical stage and tumor location, we also compared BMI1 expression in stages 1–2 tumor samples with stages 3–4 tumor samples, and the glottic tumors with the supraglottic tumors. The results revealed that BMI1 expression in stages 3–4 tumors was approximately twice that in stages 1–2 tumors (p < 0.05) (Fig. 1Bii), and there was a non-significant difference in BMI1 expression between glottic and supraglottic tumors (Fig. 1Biii). We also performed Western blot analysis to examine BMI1 expression at the protein level (Fig. 1Ci). The results were consistent with those of real-time PCR and revealed that BMI1 was highly expressed in primary tumors compared with paracancerous tissues (p < 0.01) (Fig. 1Cii) and that BMI1 protein expression was higher in stages 3–4 tumors (Fig. 1Ciii).

Establishment of a BMI1 downregulated stable cell line

To study the biological role of BMI1 in laryngeal cancer, a BMI1 shRNA stable cell line was established. As shown in Fig. 2A, the double-stranded target sequences were inserted into MluI and Clal cloning sites, which were regulated by the H1 promoter, and the interference plasmid also expressed green fluorescent protein (GFP) regulated by the EF1-alpha promoter. After transfection of 293T cells for 72 h, the cellular supernatant was collected to transfect the target cells (Fig. 2B). Hep-2 cells were transfected with shRNA and after 48 h the Hep-2 cells with BMI1 knockdown were observed under a fluorescence microscope to confirm stable interference (Fig. 2C). Real-time PCR revealed that the expression of BMI1 was dramatically reduced in RNA-interference cells and the interference rate exceeded 80% (Fig. 2D).

BMI1 knockdown inhibited Hep-2 cell proliferation and promoted cell death in vitro

To evaluate proliferation capacity, Hep-2 cells and RNA-interference cells were cultured in 96-well plates, and the absorbance of the cells was measured by the CCK-8 method on days 1, 3, 5, and 7. As shown in Table 1 and Fig. 3A, the proliferation capacity of RNA-interference cells was much lower than that of Hep-2 cells from the third day and started to decline from the fifth day. In contrast, the proliferation capacity of Hep-2 cells showed an obvious trend of increase every day. The results of colony formation assays confirmed the influence of BMI1 on proliferation capacity. The percentage of the Hep2 cell colony area was 16.455 ± 4.958%; in contrast, the percentage of the RNA-interference cell colony area was 4.958 ± 1.834% (p < 0.01) (Fig. 3B). To evaluate the effect of BMI1 knockdown on the induction of apoptosis, both Hep2 and RNA-interference cells were stained with 50 μl TUNEL reaction mixture and observed under a fluorescent microscope. As Fig. 3Ci shows, RNA-interference cells exhibited more apoptosis. The cells were also stained with 50 μg/ml PI, and the apoptotic rate was analyzed by flow cytometry. The apoptotic rate among the RNA-interference
cells was 13.34 ± 0.84%, compared with 5.81 ± 0.18% among the Hep2 cells (p < 0.01) (Fig. 3Cii and Ciii).

BMI1 shRNA blocked cell cycle distribution at the G0/G1 phase in Hep-2 cells

A cell cycle assay was performed to evaluate the cell cycle distribution by flow cytometry, in order to determine whether the inhibition of cell growth was related to cell cycle arrest. Fig. 4A shows representative DNA distribution histograms, and a comparison of the number of cells in different phases is given in Fig. 4B. RNA-interference cells showed an obvious accumulation in G0/G1 phase (p < 0.01) that was accompanied by a decrease in S phase (p < 0.01) and G2/M phase (p < 0.01).

BMI1 shRNA increased the radiotherapy sensitivity

A radiation sensitivity assay was performed to evaluate whether the knockdown of BMI1 increased the radiation sensitivity, providing a possible treatment strategy for patients with laryngeal squamous cell carcinomas. (A) Collected laryngeal tumor samples were immunolabeled with antibody BMI1 (red). The nuclei were stained with DAPI (blue). The immunofluorescence was observed under fluorescence microscope (magnification ×200). (B) The evaluation of BMI1 expression using real-time-PCR. (i) The comparison of 20 tumor samples with 20 paracancerous epithelial tissues. Results were mean ± SD (n = 20; p < 0.01). (ii) The comparison of six stages 1–2 tumor samples with fourteen stages 3–4 tumor samples. Results were mean ± SD (p < 0.01). (iii) The comparison of four glottic tumor samples with 16 supraglottic tumor samples. Results were mean ± SD (p < 0.01). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
geal carcinomas. Both Hep2 and RNA-interference cells were irradiated with doses of 2, 4, or 8 Gy. After 48 h, the growth inhibition rates of RNA-interference cells increased with increasing IR dose and were much higher than the inhibition rates of Hep2 cells ($p < 0.01; **p < 0.05; ***p < 0.01$) (Fig. 5 and Table 2).

**BMI1 silencing enhanced the sensitization to cisplatin**

To evaluate the sensitivity of Hep2 and RNA-interference cells to the chemotherapeutic drug cisplatin, the CCK-8 method was applied after 24 h incubation with cisplatin, at doses of 3, 6, and 12 $\mu$g/ml. As Fig. 6 and Table 3 show, the growth inhibition rates of both Hep2 and RNA-interference cells increased with increasing cisplatin dose. More important, the inhibition rates of RNA-interference cells were significantly higher than those of Hep2 cells with all three doses of cisplatin ($p < 0.01; **p < 0.01; ***p < 0.01$. These results indicate that BMI1 silencing enhanced the sensitivity of Hep2 cells to cisplatin, which might provide new hope to solve chemotherapeutic resistance of laryngeal cancer.

**BMI1 knockdown suppressed tumorigenesis in vivo**

To evaluate whether downregulation of BMI1 could inhibit the tumorigenic capacity of Hep2 cells in vivo, Hep2 and RNA-interference cells were injected into the subcutaneous space of the axillary fossa of mice under anesthesia. After 8 weeks, all mice were sacrificed by cervical dislocation, and tumors were isolated and weighed (Fig. 7A). We observed a significant reduction in the mean tumor weight in the BMI1 knockdown group compared with the Hep2 control group ($p < 0.01$), as shown in Fig. 7B. The weight of Hep2-formed tumors was 0.178 ± 0.008 g, and the weight of RNAi-formed tumors was 0.038 ± 0.007 g. Moreover, hematoxylin-eosin staining confirmed the histological characteristics of the tumor nodules (Fig. 7C). Therefore, downregulation of BMI1 could repress the ability of Hep2 cells to form tumors in vivo.

**Figure 2. BMI1 was knocked down mediated by shRNA interference.** (A) Schematic diagram of interference plasmid. The target fragments inserted into MluI and ClaI sites, which was regulated by H1 promoter. The expression of GFP was regulated by EF1-alpha promoter. (B) The transfected 293T cells were collected and observed under fluorescence microscope after 48 h (magnification ×200). (C) Hep2 cells were transfected and the RNA-interference cells which obtained GFP protein (green) were observed under fluorescence microscope (scale bars, 5.0 $\mu$m). (D) The interference rate was evaluated by real-time PCR, which compared BMI1 expression in Hep2 and RNA-interference cells. Results were mean ± SD ($n$ = 3; $p < 0.01$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Discussion

As one of the most common and severe malignancies of the head and neck, laryngeal SCC remains incurable at advanced stages. Therefore, we particularly wanted to understand the pathogenesis on a genetic level, which might give a new insight into how to treat the patients. The family of polycomb group (PcG) proteins comprises chromatin-modifying proteins that play important roles in embryonic development and cancer progression. BMI1, the first PcG gene to be identified, was reported to be extensively upregulated in a variety of malignancies. However, the mechanisms underlying laryngeal SCC have not been revealed. Here we demonstrated for the first time that BMI1 is highly expressed in primary laryngeal tumors and that knockdown of endogenous

Figure 3 BMI1 down-regulation suppressed the proliferation and promoted apoptosis. (A) The proliferation assay was performed on both Hep2 and RNA-interference cells. The absorbance was measured on days 1, 3, 5, and 7 according to CCK-8 method. (B) Colony formation assay was also performed to assess the proliferation capacity. (i) In each well, only one Hep2 or RNA-interference cell was allowed to grow for 2 weeks. The toluidine blue stained colonies were observed and measured under microscope (magnification × 160). (ii) Comparing with the fixed measuring-frame, the percentage of colony area formed by Hep2 and RNA-interference cell was calculated. Results were mean ± SD (n = 8; *p < 0.01). (C) Apoptosis assays assessed the impact of BMI1 knockdown on apoptosis. (i) Both Hep2 and RNA-interference cells were stained with TUNEL (green, the apoptotic cells) and DAPI (blue, nuclei). The photographs were taken under fluorescent microscope (magnification × 400). (ii) The cells were stained with PI and the apoptosis rate was evaluated by flow cytometry quantitatively. The figure showed a representative result. (iii) The comparison of apoptosis rate between Hep2 and RNA-interference cells. The result of Hep2 cells was 5.81 ± 0.183% and the result of RNA-interference cells was 13.34 ± 0.841% (n = 3; *p < 0.01). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Figure 4 BMI1 silencing induced the accumulation of G0/G1 phase. (A) Cell cycle assays were performed to assess the influence of BMI1 down-regulation on the cell cycle. (i) The representative cell cycle result of Hep2 cells, in which the G0/G1 phase was 64.31%. (ii) The representative result of RNA-interference cells, in which the G0/G1 phase was 87.41%. (B) The comparison of the cells in G0/G1, S and G2/M phases between Hep2 and RNA-interference cells. Results were mean ± SD (n = 3; *p < 0.01; **p < 0.01; ***p < 0.01).
BMI1 down-regulation increased the radiotherapy sensitivity. Both Hep2 and RNA-interference cells were treated with 2, 4, and 8 Gy radiotherapy and cultured for 48 h. The inhibition rates were measured and calculated based on CCK-8 method. Results were mean ± SD (n = 3; *p < 0.01; **p < 0.05; ***p < 0.05).

Table 2: Inhibition Rate of Hep2 and RNAi cells to radiotherapy.

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<td>0.158 ± 0.010 * *</td>
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* p < 0.01,
** p < 0.05,
*** p < 0.05 (compared with RNAi cells with the same doses).

BMI1 expression suppresses proliferation and promotes apoptosis, thus sensitizing laryngeal Hep2 cells to the anticancer drug cisplatin and radiotherapy.

In this study, we collected 20 primary laryngeal SCC specimens and 20 paracancerous epithelial tissue samples. Immunofluorescent staining indicated that BMI1 was highly expressed in the tumor samples. In most of the tumor samples, BMI1 could be observed at the basal layer and the invasive front of tumor cell nests, as the basal cell layer represents a compartment of cells with a high proliferative capacity necessary for the self renewal of the epidermis; this is consistent with Vormittag's results. However, we found that in some tumor samples, BMI1 was also highly expressed in the tumor nests, which might be related to the extent of differentiation since expression of BMI1 was stronger in highly differentiated tumor cells than in poorly differentiated tumor cells. To evaluate the expression of BMI1 quantitatively, we performed real-time PCR, and the results revealed that BMI1 expression was much higher in primary tumors than in paracancerous tissues. Interestingly, we also observed that BMI1 expression in stages 3–4 tumors was approximately twice that in stages 1–2 tumors, and there was a non-statistically significant difference in BMI1 expression between glottic and supraglottic tumors. This indicated that the expression of BMI1 was correlated with the progression of laryngeal carcinoma, thus influencing the prognosis as previous studies have indicated in colon, tongue, and breast carcinomas. However, there was no evidence of a relationship with the tumor location. Further, the signal transduction pathway of BMI1 in tumor progression has not been revealed and is still under investigation.

A cell proliferation assay was used to investigate whether the downregulation of BMI1 influenced the growth of Hep2 cells in vitro. By CCK-8 methods, we showed that the proliferation of RNA-interference cells was much slower than that of Hep2 cells and RNA-interference cells and even started to cease proliferation from the third day. The results of colony formation assays also revealed that RNA-interference cells had a lower capability to form colonies. Previous studies reported that BMI1 can regulate normal cell proliferation and that BMI1 knockout leukemic cells showed proliferation arrest. More recently, Song et al. reported that BMI1 is related to proliferation, survival, and poor prognosis in pancreatic cancer. Therefore, our results are consistent with the previous report that BMI1 knockdown may repress proliferation of Hep2 cells. Moreover, we confirmed this finding by a tumorigenesis assay in vivo, which indicated that RNA-interference cells were less able to develop into tumors. Recently it has been reported that BMI1 is associated with the protection of tumor cells from apoptosis. These observations prompted us to investigate the effect of BMI1 knockdown on the induction of apoptosis, and we found that RNA-interference cells were more likely to undergo apoptosis, consistent with Qin's findings in nasopharyngeal carcinoma cells. Therefore, our results indicate that BMI1 plays significant roles in the proliferation and apoptosis of laryngeal Hep2 cells.

The cell cycle is closely related to cell proliferation and apoptosis, and it has previously been reported that BMI1 controls the cell
To determine the mechanism by which BMI1 knockdown suppresses Hep2 cell proliferation and promotes apoptosis, cell cycle assays were performed. It has been reported that BMI1 knockout leukemic cells displayed accumulation at the G0/G1 phase with fewer cells in S phase, and lung cancer A549 cells transfected with antisense BMI1 displayed increase at the G0/G1 phase and reduction at the S phase. Additionally, HeLa cells transfected with BMI1 siRNA showed accumulation at the G1 phase and demonstrated G0/G1 phase arrest with a decrease at the S phase, prevention of mitosis, and ultimately inhibition of cell proliferation. However, Cui et al. reported a conflicting result in that BMI-1 knockdown had no significant effect on cell cycle status in human neuroblastoma I BE(2)-C cells. In the present study, cell cycle assays revealed that downregulation of BMI1 led to an obvious increase in cells in G0/G1 phase that was accompanied by a reduction of cells in S phase and G2/M phase, which indicates inhibition of mitosis and cell proliferation. Thus, BMI1 maintained the proliferation capacity of laryngeal Hep2 cells through a mechanism that might be determined by cell cycle regulation.

To determine the relationship between downregulation of BMI1 and radiochemotherapy sensitivity, we performed radiotherapy and chemotherapy sensitivity assays. The results revealed that...
BM1 knockdown led to a significant enhancement of radiotherapy and chemotherapy sensitivity compared with Hep2 cells. According to the study of Alajez et al., BM1 showed a radiosensitivity function in nasopharyngeal cancer (NPC), whereby BM1 depletion sensitized NPCs to radiotherapy. Additionally, Chen et al. reported that BM1 was a potential target for increasing the sensitivity of head and neck SCC cancer stem cells, which originated from oral carcinomas, to chemoradiotherapy. Our findings were consistent with previous studies and may provide new hope to patients with laryngeal SCC. Since BM1 is considered to be a stem cell-related gene and has been implicated in multiple cancers, BM1 might be a target of laryngeal cancer stem cells that plays a significant role in radiochemotherapy resistance, although further investigations are still needed. Moreover, since the functions of BM1 are mainly attributed to its repressive action on the INK4A/ARF locus and on p53 activity, and defects in apoptosis due to p53 inactivation could result in treatment-resistant tumors, BM1 may regulate alternate cellular pathways to promote treatment-resistant tumor formation. Furthermore, inhibition of PI3K activity has been shown to enhance the effects of chemotherapeutic agents and depletion of BM1 expression was observed to reduce PI3K/AKT activity. Hence, the BM1-regulated PI3K/AKT pathway might be involved in the radiochemotherapeutic resistance of laryngeal tumors. We intend to perform further investigations to clarify this.

In conclusion, we report for the first time that BM1 is highly expressed in laryngeal SCC and is significantly associated with tumor progression. Furthermore, we demonstrate that depletion of BM1 inhibits proliferation capacity and promotes apoptosis of laryngeal Hep2 cells due to accumulation of cells in G0/G1 and sub-G0 phases of the cell cycle. Additionally, down-regulation of BM1 reduces radiochemotherapeutic resistance, suggesting an essential role for BM1 in the treatment of patients with laryngeal carcinoma. Taken together, our results suggest that depletion of BM1 is a potential therapeutic option for cancer treatment.

Conflict of interest statement
None conflict of interests.

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Reference