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Original Article

Bmi-1 serves as a potential novel marker for progression in human cutaneous basal cell carcinoma

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Abstract: B-cell-specific Moloney murine leukemia virus integration site 1 (Bmi-1) is one of the core members of the PRC1 complex (the polycomb gene family) involved in tumorigenesis. The aim of this study is to investigate the expression and possible function of Bmi-1 in cutaneous basal cell carcinoma (BCC). Here, by immunohistochemical staining, we found that elevated Bmi-1 expression was more commonly observed in fresh BCC tissues compared with normal. Bmi-1 RNAi revealed that suppression of endogenous Bmi-1 expression in A431 cells attenuated cell proliferation, invasion, and induced apoptosis of BCC cells. Indicating Bmi-1 may play an important role in progression of BCC and may serve as a novel marker for therapeutic targets.

Keywords: BMI-1, basal cell carcinoma, proliferation, invasion, apoptosis

Introduction

Basal cell carcinoma is the most common type of skin cancer and its incidence is increasing worldwide [1, 2]. It develops from the basal layers of the epidermis and hair follicles with the characteristics of slow growing, locally invasive and destructive and usually occurs on actinic damaged skin of older people. Despite the development of new prognostic and therapeutic strategies, including surgical resection or surgery combined with photodynamic therapy, the incidence rate of BCC is still increasing year by year. Statistics have shown that BCC accounted for 80% of nonmelanoma skin cancers [3]. Therefore, a better understanding of the underlying molecular events leading to the initiation and progression of BCC could result in the identification of potential diagnostic and therapeutic targets.

Bmi-1 is one of the core members of the polycomb repressive complex 1 (PRC1 complex), was identified as an oncogene, which plays an important role in cell immortalization and cell senescence [4]. Bmi-1 possibly through a variety of mechanisms mediated tumorigenesis and development, and the influence in different tumors are vary. Previous studies have reported that Bmi-1 is over-expressed in many human malignancies, including hepatocellular carcinoma, gastric, lung, leukemia, colon, glioma, ovary, malignant melanoma, lymphoma [5-13]. And high Bmi-1 expression has been reported to be associated with an adverse prognosis in multiple type of cancer [14]. But in different human malignancies, Bmi-1's roles are various. In nasopharyngeal carcinoma, Bmi-1 may enhance the motility and invasiveness of human nasopharyngeal epithelial cells, via modulation of PI3K/Akt/GSK-3beta signaling pathway [15]. Previous studies suggest that Bmi-1 promotes glioma cell migration and invasion via NF-κB-mediated upregulation of MMP-3 [16]. However, Bmi-1 might modulate the growth of lung adenocarcinoma cells in an INK4a-p16 independent pathway [17]. Taken together, Bmi-1 protein plays an important role in human cancers.

However, little is known about Bmi-1 expression in basal cell carcinoma. In this study, we investigated the expression pattern of Bmi-1 in BCC and explored the role of Bmi-1 in the human epidermal carcinoma cell line (A431).

Materials and methods

Specimens

Seventy-six paraffin-embedded tissue specimens of BCC were obtained from department of Dermatology and Venereology, Affiliated Ho-

spital of Nantong University between 2014.01 and 2015.03. All of patients had not received therapy before surgery. Histopathological examination of the specimens was confirmed by three pathologists independently. Twenty specimens of normal skin (N) were used as controls. Ten fresh BCC and normal skin specimens were transported into liquid nitrogen and stored immediately at -80°C after surgery until use. The study approval was obtained from the ethics committee of Nantong University, Affiliated Hospital.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from tissues with Trizol reagent (Invitrogen, Carlsbad, CA, USA), and the reverse transcription reactions were performed with random primers and revertaid first strand cDNA synthesis kit (Thermo) following the manufacturer's instructions. Real-time PCR was performed by using SYBR Green Master Mix (Rox, Weitefeld, Germany) protocol on 7500 Real-Time PCR System (ABI, Abilene, TX, USA) according to the instructions. Primer sequences used were as follows: Bmi-1, forward 5'-AGAGATCGGGGCGAGACAAT-3' and reverse 5'-TTATTCTGCGGGGCTGGGAG-3'; forward 5'-AGTATGAGAGGCAGAGATCGGG-3' and reverse 5'-GAGGTGCTGCTAAGCCGGAA-3'. GAPDH (glyceraldehyde-3-phosphate-dehydrogenase) forward, 5'-TCG GAG TCA ACG GAT TTG GTC GT-3'; GAPDH reverse, 5'-TGC CAT GGG TGG AAT CAT ATT GGA-3'; GAPDH was used as references for Bmi-1. All the experiments were repeated in triplicate. The 2-ΔΔCt method was used to determine the relative quantitation of gene expression levels.

Western blotting

The protein was extracted from frozen tissues and cultured cells using RIPA lysis buffer containing phosphatase inhibitor (100:1). The lysates were centrifuged at 14000 rpm for 20 min at 4°C. The protein concentration was determined with a Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). The protein was transferred onto polyvinylidene difluoride filter (PVDF) membrane after separating by 10% SDS-PAGE, and then incubated overnight at 4°C with antibody against Bmi-1 (CST, TX, 1:1000) and Beta-actin (Santa Cruz Biotechnology, USA). PVDF membranes were incubated with secondary antibodies at room temperature for 2 h. Bound proteins were scanned by using Chemi Doc XRS (Hercules, CA). Bound proteins were detected by using ECL (enhanced chemiluminescence) detection systems (Pierce, Rockford, IL, USA).

Immunohistochemistry

Xylene and rehydrated were used to deal with the sections. The sections were deparaffinized using a graded ethanol series, and then endogenous peroxidase activity was blocked by soaking in 3% hydrogen peroxide for 30 min. Antigen retrieval was carried out by boiling sections in 10 mM citrate buffer (pH 6.0), and 10% normal goat serum was used for blocking any nonspecific reactions. The sections were immunostained for Bmi-1 using rabbit anti-human Bmi-1 antibody (1:600), and biotinylated goat antirabbit serum IgG was used as secondary antibody. Diaminobenzidine (DAB) served as chromogen. All immunostained sections were randomly examined by two independent blinded pathologists using a Leica fluorescence microscope (Germany).

Cell line culture

The A431 cells were provided by Institute of Dermatology, Chinese Academy of Medical Sciences and Peking Union Medical College and were cultured in Dulbecco's modified Eagle medium (DMEM, GIBCO-BRL, Grand Island, NY, USA), with 10% fetal bovine serum (FBS) and 1% antibiotics (both from Gibco, USA). Cells were incubated in humidified incubator under the condition of 37°C, 5% CO_2.

Immunofluorescence analysis

The cells were cultured overnight on preferred glass coverslips (Fisher, Pittsburgh, PA). The cells were fixed for 30 min in 4% paraformaldehyde (Electron Microscopy Services, Ft. Washington, PA), permeabilized with 0.1% Triton X-100/PBS for 15 min, and then incubated for 2 h in a 1% BSA/PBS blocking solution. To detect Bmi-1, a rabbit polyclonal antibody was used (Cell Signaling Technology, USA; 1:100 dilution) and incubated overnight at 4°C. The secondary antibody was performed using Alexa Fluor 568-conjugated goat anti-rabbit antibodies (Invitrogen) for 2 h at 37°C. To visualize nuclei, cells were stained with 5 mg/ml DAPI (Sigma) for 3 min. All images were captured with a Nikon confocal microscope (Nikon, NY, USA).

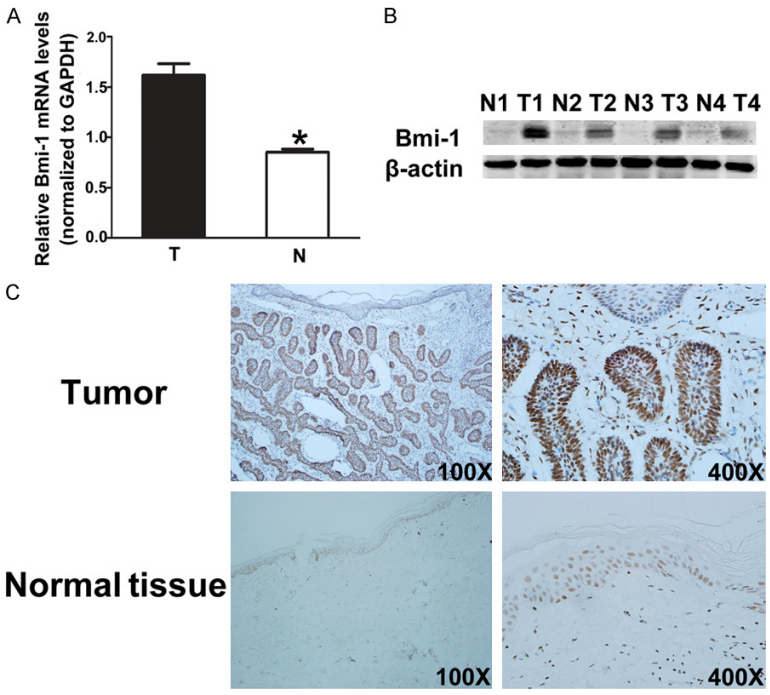

Figure 1. Bmi-1 was upregulated in BCC tissues. A. Bmi-1 levels were evaluated by qRT-PCR in four tissues, and normal human tissues were used as controls (*P<0.05). B. Bmi-1 and β-actin protein in the four BCC tissues and normal human tissues analyzed by Western blot analysis (*P<0.05). C. Bmi-1 expression and distribution was examined by Immunohistochemistry in BCC and normal tissues. Bmi-1 was highly expressed in BCC tissue, whereas low expression of Bmi-1 was observed in normal tissues. The experiment details were described under Materials and Methods section.

Silencing of Bmi-1

Four short hairpin RNA (shRNA) duplexes targeting Bmi-1 and control scramble shRNA duplexes were purchased from GeneChem (Shanghai, China). The effective sequences were as follows: 5'-TAATACTTTCCAGATTGAT-3' (shRNA#1). Another sequences list in the table. The nonspecific scramble shRNA duplex was 5'-TTCTCCGAACGTGTCACGT-3', which was the random sequence that was not related to Bmi-1 mRNA. A431 cells were plated onto a 6-well plate or a 96-well plate (Corning Inc., Corning, NY, USA) at 60-70% confluence the day before transfection. After 24 h, the Bmi-1-targeting and control shRNA oligos were transfected into the cells using Lipotransfectamine 2000 (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions.

shRNA#2	5'-GAAAGTAAACAAAGACAAA-3'
shRNA#3	5'-AGAACAGATTGGATCGGAA-3'
shRNA#4	5'-AGACCACTACTGAATATAA-3'

Cell invasion assay

The invasive potential of cells was evaluated using 24-well cell culture with 8 μm pores (Coring, USA). For invasion assay, 1×10^5 A431 cells (transfection of control shRNA or Bmi-1 shRNA) in a 100 μl volume of serum-free medium were seeded into insert chamber pre-coated with Matrigel matrix (BD, NJ, USA), and a volume of 0.5 mL 10% FBS medium was added to the matched lower chamber. Chambers were incubated at 37°C under 5% CO_2 condition for 48 h. Invasive cells on the lower surface of the membrane, which had invaded the Matrigel and had migrated through the polycarbonate membrane, were fixed in 4% formaldehyde for 30 min and stained by crystal violet for 30 min.

Cell proliferation assay

A431 cells transfected with Bmi-1-shRNA and negative control-shRNA were seeded into 96-well cell culture cluster plates (Corning, USA) at a concentration of 2×10^4 cells/well in volumes of 100 μl complete medium and cultured overnight at 37°C. For proliferation measurement, 10 μL CCK-8 reagents (Dojindo, Kumamoto, Japan) was added to each well at different time points and then incubated for another 1.5 h at 37°C in the dark. The absorbance of cells was read in a microplate reader (Bio-Rad, USA) at 450 nm. The detection of cell absorbance was performed every 24 h.

Plate colony formation assay

A431 cells (transfection of control shRNA or Bmi-1-shRNA) were added to each well of a 6-well culture plate and cultured in 5 ml of DMEM supplemented with 10% FBS. After 2 weeks of incubation, cell colonies were washed twice with PBS, fixed with 4% para-formaldehyde for 30 min and then stained with 0.5%

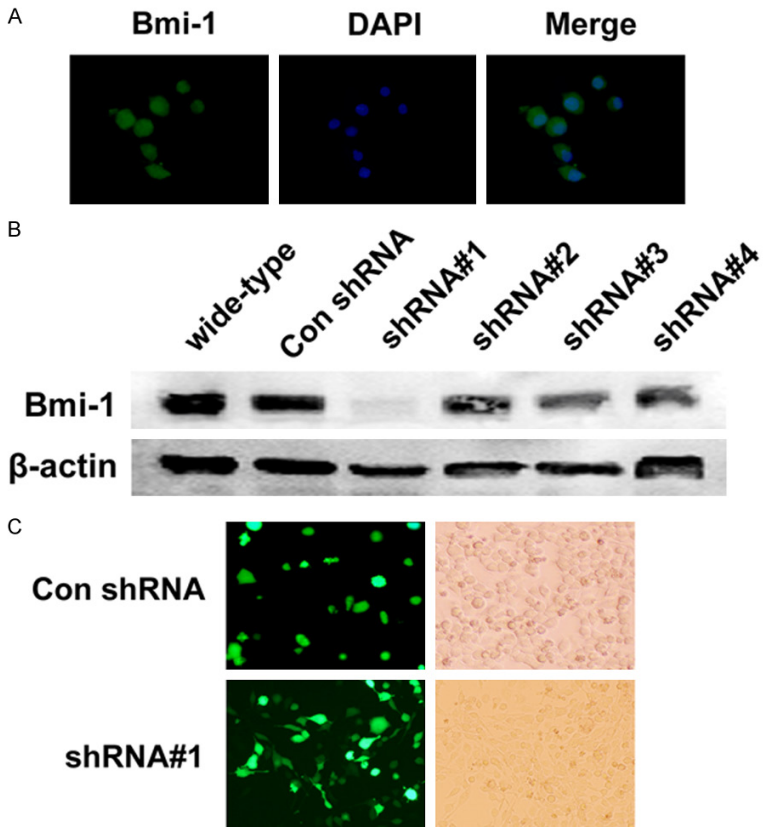

Figure 2. A. Immunofluorescence analysis of Bmi-1 expression in A431 cell line. Bmi-1 was dominantly expressed in the cytoplasm and nucleus of the A431 cell line. B. Bmi-1 expression was detected by Western blot after transfected for 48 h with Bmi-1-shRNA in A431 cells, while shRNA#1 achieved the more effected down-regulation. The relative level of Bmi-1 was tested by densitometry. The date are mean ± SEM *P<0.05 compared with the control. C. Fluorescence observation of the transfection efficiency shRNA-GFP in A431 cell line (magnification ×100).

crystal violet for 30 min. Individual clones with >0.5 mm were counted as positive for growth.

Flow cytometry analysis of cell apoptosis

A431 cells transfected with Bmi-1-shRNA and control shRNA were cultured for 48 h and digested by 0.1% trypsin. Then cells were washed with cold PBS and resuspended in 1× Binding Buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) at a concentration of 1×10^6 cells/ml. Transfer 100 μl of the solution (1×10^5 cells) to a 5-ml culture tube. For analysis of the cell apoptosis, cells were incubated with reagent containing Annexin V and PI (Becton-Dickinson, NJ, USA) for 15 min at room temperature in the dark. After 15-min incubation in darkness at room temperature, add 300 μl of 1× Binding Buffer to each tube. Subsequently, Apoptotic cells were analyzed using FACSCalibur flow cytometer (Becton-Dickinson, NJ, USA).

Statistical analysis

Statistical analysis was performed using the Stat View 7.0 software package. The SPSS 21.0 and SigmaPlot 12.0 statistical program was employed to conduct statistical analysis for all statistical analyses. The relationship between Bmi-1 and clinicopathological features was evaluated using chi-square tests. The data of *proliferation*, invasion and *apoptosis* were analyzed with the student's t-test. The data are presented as means ± SEM from at least three separate experiments. P<0.05 was considered statistically significant.

Results

Elevated Bmi-1 expression in BCC tissues

To determine whether Bmi-1 was expressed in the tissue of BCC, We firstly analyzed Bmi-1 mRNA expression in four BCC tissues and corresponding normal tissues, the expression of Bmi-1 mRNA was significantly higher in BCC tissues than that in corresponding normal tissues (**Figure 1A**, P<0.05). Analogously result was also found in Western blot analysis, when compared with the normal tissues, Bmi-1 protein was significantly up-regulated (**Figure 1B**). Immunohistochemical method was used to explore the expression pattern of Bmi-1 in BCC (**Figure 1C**). In accordance with the expression profile of Bmi-1 in fresh tissues, strong positive staining of Bmi-1 was found in BCC, distributed both in nuclear of tumor cells. Whereas, normal specimens showed weak staining (**Figure 1C**).

Expression and silencing of Bmi-1 in A431 cell line

The A431 which is widely recognized as a model of basal cell carcinoma. So A431 cell line was

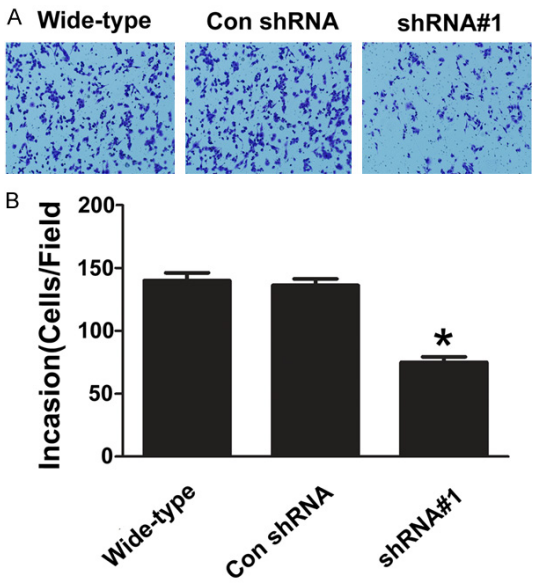

Figure 3. Knockdown of Bmi-1 suppressed the invasion of A431 cells. A431 cells invasion were evaluated by Transwell invasion assays after transfected with Bmi-1-shRNA, and the results showed that cell invasion was significantly decreased compared with the shRNA control. Quantification of cell invasion is shown on the below panel. All data are representative of three independent experiments (mean ± SEM) (*P<0.05).

chosen to study the biological functions of endogenous Bmi-1. To observe the expression happened in Bmi-1, we measured the Bmi-1 level in BCC cell line. Expression of Bmi-1 protein was verified by both immunofluorescence and western blot (**Figure 2A**, **2B**). In A431 cell line, Bmi-1 significantly located in cytoplasm and nuclear of tumor cells (**Figure 2A**). To better investigate the biological functions of endogenous Bmi-1 in A431 cells, we used a short hairpin RNA-oligo targeting Bmi-1 to knock down the endogenous expression of Bmi-1 in A431 cells. The Bmi-1 levels in shRNA transfected cells were determined by western blot observation. A knockdown effect was discovered by western blot, and we found that among the four targeting (shRNA#1-4) shRNA#1 achieved the greater efficacy in silencing Bmi-1 expression compared to the negative control (Wide-type and Con-shRNA) (**Figure 2B**). Immunofluorescence observation revealed that most of the A431 cells expressed GFP at a relatively high level (**Figure 2C**).

Knock-down of Bmi-1 inhibited the invasion in vitro

Cell invasion is integral step for the process of tumor development and metastasis. To examine the effect of Bmi-1 on A431 cell invasion, we used a Matrigel invasion assay. With a chamber pre-coated with Matrigel, we determined changes in cell invasiveness after 48 h incubation. Compared with the Con-shRNA cells, Bmi-1-shRNA cells revealed significantly decreased invasiveness (**Figure 3B**). The result suggested that Bmi-1 promoted the invasion of A431 cell line.

Depletion of Bmi-1 restrained cell proliferation and induced apoptosis

Effect of decreased Bmi-1 expression on A431 cell proliferation in vitro was examined by CCK-8 assay. Silencing of Bmi-1 caused significantly decrease in cell viability in A431 cells (**Figure 4A**). Plate colony formation assay also showed similar results (**Figure 4B**). In addition, we examined the effect of Bmi-1 on BCC cells' survival. A Flow cytometry analysis was performed to investigate whether Bmi-1 inhibited apoptosis in A431 cells. When Bmi-1 was knocked down in A431 cells, cell apoptosis was significantly increased compared with both Wide-type and control shRNA-transfected cells (**Figure 4C**). Taken the above results together, we guess that Bmi-1 might promote A431 cell proliferation and inhibited apoptosis.

Discussion

The polycomb group (PcG) genes play a centrally important role in mammalian development. Bmi-1 is the mammalian form of a Drosophila PcG protein that regulates stem cell maintenance and renewal [18]. Previous studies have reported that Bmi-1 is over-expressed in many human cancers. A high expression of Bmi-1 positively correlated with poor prognosis [19]; And overexpression of Bmi-1 induced malignant transformation both in vitro and in vivo [20]. Similarly Bmi-1 also expression in many skin diseases, such as malignant melanoma, squamous cell carcinoma, mycosis fungoides, Merkel cell carcinoma [4, 18, 21, 22], etc. These findings suggest that Bmi-1 may play a role in the development and maintenance of hyperproliferative skin diseases, and is consistent with the observation that Bmi-1 is overexpressed in other tumor types. However, Bmi-1 expression as well as its function has not yet been established in BCC.

In our study, we demonstrated for the expression level of Bmi-1 which was expressed re-

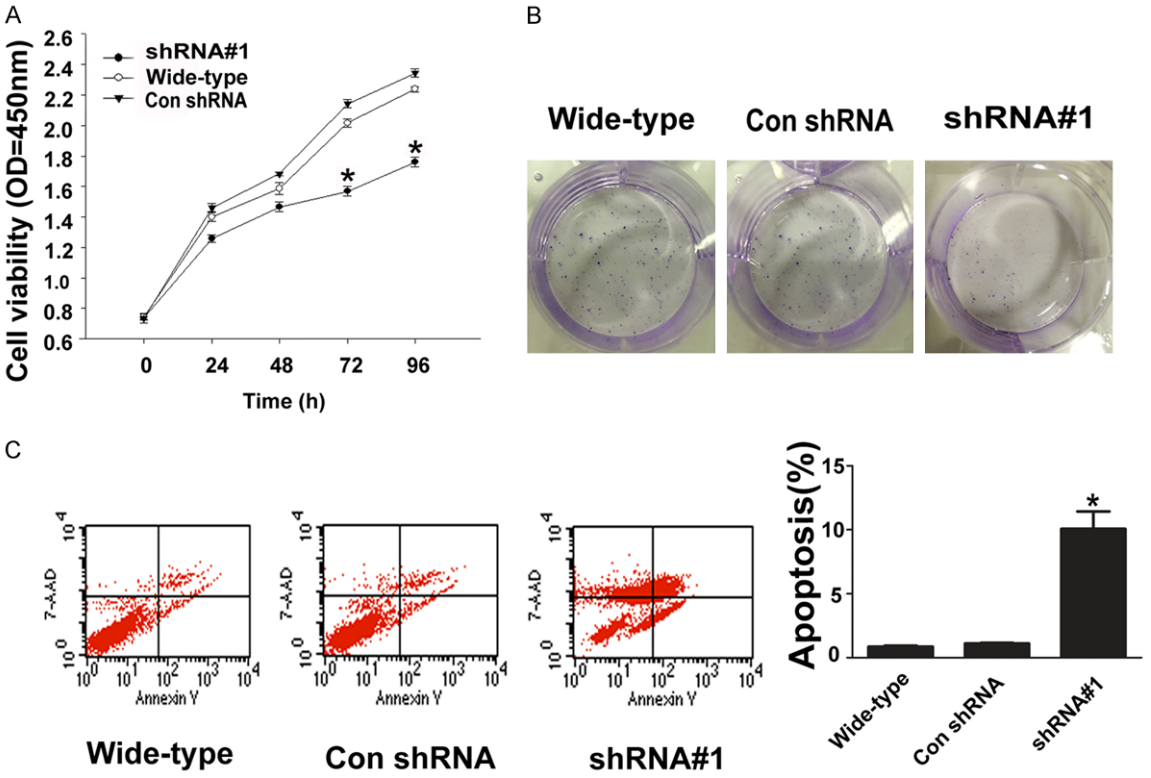

Figure 4. Knockdown of Bmi-1 suppressed A431 cell proliferation. A. After 48 h transfection, CCK-8 assay was performed to determine the proliferation of A431 cells. B. Bmi1 knockdown suppressed plate colony formation. C. At 48 h after transfection, the apoptotic A431 cells were detected by flow cytometry. The data are mean ± SEM. *P<0.05, when compared with controls. All data are representative of three independent experiments (mean ± SEM) (*P<0.05).

markably high in fresh BCC specimens by using western blot assays and qRT-PCR assays. In order to reveal the relationship between the Bmi-1 protein expression and the progression of BCC, we investigated Bmi-1 expression in BCC using immunohistochemical staining. Our findings discovered that the expression of Bmi-1 protein was significantly higher in BCC than that in normal specimens. These results suggested that Bmi-1 protein may be a maker of BCC. This was in line with some previous studies, for instance, measuring Bmi-1 autoantibody levels of patients with cervical cancer could have clinical prognostic value as well as a non-tissue specific biomarker for neoplasms expressing Bmi-1 [23].

Previous reports have shown that Bmi-1 mRNA is readily detected in Keratinocyte cell lines A431. And the level of expression is markedly increased [18]. The proliferation and invasion are the important characteristics of cancer development, and apoptosis has an important role in the regulation of oncogenesis. To investigate the biological functions of Bmi-1 in BCC, we chose to use A431 cells exhibiting the expression of endogenous Bmi-1, and employed the shRNA to knock down the expression level of endogenous Bmi-1. We found suppressed Bmi-1 expression decreased the A431 cells and promotes cell apoptosis, which are both indispensable for BCC growth. These results strongly suggest an oncogenic role of Bmi-1 in the human BCC development.

The Polycomb group (PcG) proteins are epigenetic suppressors of gene expression that function through modification of histones to change chromatin structure and modulate gene expression and cell behavior [24]. Bmi-1 is a member of the Polycomb family of transcriptional repressors. It is involved in cell senescence and cell regulation by repression of Ink4a/Arf locus, encoding cell cycle regulators p16Ink4a and p19Arf [25-27]. Ink4/Arf locus is a key downstream target of Bmi-1 gene. Excision of this

locus is seen with high frequency in a great variety of malignancies including glioblastoma, melanoma, pancreatic adenocarcinoma, non-small cell lung cancer, bladder carcinoma and oropharyngeal cancer [28].

Bmi-1 can induce downstream telomerase reverse transcriptase gene expression, playing an important role in telomerase activation, cell immortality, cell senescence and neoplastic transformation [29]. Besides, Bmi-1 nuclear localization could be an important marker for the diagnosis of gastric cancer [30]. Bmi-1 as an important new target for therapy in ovarian cancer and liver cancer [31, 32]. Similar results can be found in some other tumors. Therefore, these results identify Bmi-1 as a potential target for cancer therapy.

In conclusion, we demonstrated that Bmi-1 was highly expressed in BCC tissues. Knockdown of Bmi-1 attenuated invasion of A431 cells remarkably in vitro. Moreover, Bmi-1 inhibits cell apoptosis, which ultimately promoted cell growth in BCC. Thus, identification of specific chemicals able to inhibit the expression or transcriptional activity of Bmi-1 would be a novel biomarker and therapeutic target of BCC. However, further research is required to better delineate the molecular mechanisms.

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Disclosure of conflict of interest

None.

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