Bmi-1-shRNA Inhibits the Proliferation of Lung Adenocarcinoma Cells by Blocking the G1/S Phase Through Decreasing Cyclin D1 and Increasing p21/p27 Levels

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B lymphoma Mo-MLV insertion region 1 (Bmi-1) is highly expressed in a variety of cancers and has been shown to regulate cell proliferation. The INK4a/ARF tumor suppressor gene locus is one of the major targets of Bmi-1. In the present study, we chose two lung adenocarcinoma cell lines, A549 cells (without INK4a locus) and SPC-A1 cells (with INK4a locus), to investigate if the small hairpin RNA-mediated knockdown of Bmi-1 could inhibit the proliferation of lung adenocarcinoma cells, and to delineate the possible mechanism underlying Bmi-1 modulation of cell proliferation. We also investigated the potential pathway underlying Bmi-1 regulation of lung adenocarcinoma cell proliferation in different genetic backgrounds. To this end, we used shRNA to knockdown Bmi-1 expression in lung adenocarcinoma cells, which led to inhibition of cell growth, colony formation *in vitro*, and tumorigenesis *in vivo*. In addition, phosphorylated Akt and cyclin D1 expression were downregulated, p21 and p27 levels were upregulated, and p16 expression remained unchanged in SPC-A1 cells. These data indicate that Bmi-1 might modulate the growth of lung adenocarcinoma cells in an INK4a-p16 independent pathway.

Introduction

APPROXIMATELY 85% OF LUNG CANCERS are non-small-cell lung cancers (NSCLC), and lung adenocarcinoma is the most common form of lung cancer in nonsmokers. It is well known that tumors result from sustained genetic alterations, including activation of oncogenes and inactivation or deletion of tumor suppressor genes, such as p16INK4a and p14ARF (a murine p19ARF equivalent), which are encoded by the INK4a/ARF locus. Deletion of INK4a/ARF is often seen in patients with lung adenocarcinoma (Toyooka et al., 2006). p16INK4a and p14ARF, which are encoded by the INK4a/ARF gene located at chromosome 9p21, are cyclin-dependent kinase (CDK) inhibitors that play important roles in retinoblastoma and p53 tumor suppressor pathways, respectively (Pomerantz et al., 1998).

The polycomb group (PcG) protein B lymphoma Mo-MLV (Moloney murine leukemia virus) insertion region 1 (Bmi-1) was originally isolated as an oncogene that cooperates with

c-Myc in a murine model of lymphoma (Van Lohuizen et al., 1991). Bmi-1 plays a key role in regulating the self-renewal of normal and malignant human mammary stem cells (Liu et al., 2006). Bmi-1 expression is upregulated in a variety of human cancers, including NSCLC (Kim et al., 2004; Glinsky et al., 2005; Sawa et al., 2005; Song et al., 2006; Becker et al., 2009), therefore, Bmi-1 might play an important role in tumorigenesis. Previous studies have indicated that the Bmi-1 gene regulates cell proliferation and senescence mainly through the INK4a locus (Jacobs et al., 1999). However, a previous study from our research group found that an antisense Bmi-1 expression plasmid inhibited the growth rates of A549 human lung adenocarcinoma epithelial cells (Yu et al., 2007), which harbor a homozygous deletion of the INK4a/ARF locus (Okamoto et al., 1995). Therefore, we hypothesized that another signaling pathway may be involved in Bmi-1 modulation of lung adenocarcinoma cell proliferation.

In this study, we investigated the potential pathway underlying Bmi-1 modulation of lung adenocarcinoma cell

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proliferation. To this end, we stably transduced A549/SPC-A1 cells with Bmi-1–small hairpin RNA (Bmi-1-shRNA) (Meng et al., 2012). Our data showed that Bmi-1-shRNA inhibited the proliferation of A549/SPC-A1 cells both *in vitro* and *in vivo*. We also explored the potential mechanism underlying the observed effects of Bmi-1 knockdown on the proliferation of lung adenocarcinoma cells in different genetic backgrounds.

Materials and Methods

Cell culture and stably transduced A549/SPC-A1 cells

A549/SPC-A1 cells were cultured in RPMI-1640 medium (Gibco) supplemented with 10% bovine calf serum (Gibco) in a humidified chamber at 37°C with 5% carbon dioxide. The construction and transduction of Bmi-1 small interference RNA (siRNA) was previously described (Meng et al., 2012). Cells transduced with PSUPER-retro-neo-Bmi-1 or a random sequence were named A549/SPC-A1-shRNA-Bmi-1 and A549/SPC-A1-ctr, respectively; untransduced A549/SPC-A1 cells were named A549/SPC-A1-wt.

Western blotting

Protein extracts were obtained by using the total protein extraction kit (KEYGEN Co. Ltd.). Protein concentrations of the supernatants were determined using the BCA Protein Assay Kit (Pierce). A total of 60 µg protein was resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electrophoretically transferred to polyvinylidene difluoride membranes (Millipore). After blocking in 5% (w/v) nonfat milk and washing in Tris-buffered saline-tween solution (TBST), the membranes were incubated with the appropriate primary antibodies at 4°C overnight. Signals were detected using an ECL Plus Western Blotting System (Amersham). The following primary antibodies were used: polyclonal anti-Bmi-1 (abcam), anti-cyclin D1 (Cell Signaling Technology), anti-p21, anti-p27, and β-actin (Santa Cruz Biotechnology).

Determination of cell viability

The effect of Bmi-1-shRNA on cell growth and viability was measured by the methyl thiazolyl tetrazolium (MTT) assay and trypan blue exclusion method. In short, cells were plated in triplicate at a density of 3×10^3 cells per well in 96-well plates. On each of 5 consecutive days, 10 µL of MTT (5 mg/mL in phosphate-buffered saline, PBS) were added to each microtiter well, and the plates were incubated at 37°C for 4 hours. After aspiration of the supernatant, 150 µL dimethyl sulfoxide was added and mixed, and the absorbance was measured with a microELISA reader (Thermo Scientific) at a wavelength of 492 nm. In addition, the cell suspension was mixed with trypan blue dye and observed under a microscope within three minutes. Trypan blue is a vital stain that leaves nonviable cells with a distinctive blue color because they take up the dye.

Colony-forming assay

A total of 300 cells in 2 mL growth medium were plated in triplicate in 6-well plates. After 10 days, the cells were rinsed twice with PBS, fixed in 10% formaldehyde, and stained with 0.1% crystal violet in 10% ethanol. The numbers of colonies were counted.

Cell cycle assay

The cell cycle was analyzed by using flow cytometry with propidium iodide (PI; Sigma) staining. In each group, the cells were harvested and washed with PBS, and fixed overnight in ice-cold 70% ethanol. The cells were washed twice with PBS and treated with 1 mg/L RNase A (Takara Co. Ltd.) for 15 minutes. Finally, the cells were stained with 50 mg/L PI in the dark for 1 hour. Cell cycle analysis was performed by flow cytometry (BD Biosciences).

In vivo tumorigenesis assay

Nude mice (Balb/c nu/nu) were obtained from the Animal Facility of Dalian Medical University (Dalian, China). For measurement of tumor growth *in vivo*, cells (1×10^7 cells/mouse) were injected subcutaneously into the armpit of nude mice ($n = 6$). After 4 weeks, tumors were isolated from nude mice, weighed, and fixed in formalin. Animal experiments were approved by The Animal Ethics Committee of Dalian Medical University (No. L2010004), and conducted according to China Public Health Service Policy on Humane Care and Use of Laboratory Animal.

Statistical analysis

Data were expressed as mean ± standard deviation and analyzed by one-way analysis of variance with SPSS software, version 13.0 (SPSS Inc.). *P*-values < 0.05 were considered statistically significant.

Results

Bmi-1-shRNA inhibits cell growth and viability

The effect of Bmi-1 knockdown on A549/SPC-A1 cell growth was determined by the MTT assay (Fig. 1A). The results indicated that the growth of A549/SPC-A1-shRNA-Bmi-1 cells was significantly slower than that of A549/SPC-A1-wt and A549/SPC-A1-ctr cells ($p < 0.05$), although there were no significant differences between A549/SPC-A1-wt and A549/SPC-A1-ctr cells ($p > 0.05$). In order to determine if the slower growth rate of A549/SPC-shRNA-Bmi-1 cells was caused by cell death, a trypan blue exclusion test was performed to assess cell viability. As shown in Fig. 1B, there were no significant differences in cell viability between A549/SPC-A1-wt A549-ctr and A549/SPCA1-shRNA-Bmi-1 cells ($p > 0.05$).

Bmi-1-shRNA decreases the clonogenicity of lung adenocarcinoma cells in vitro

Colony formation assay is an *in vitro* cell survival assay based on the ability of a single cell to grow into a colony. The colony is defined to consist of at least 50 cells. The assay essentially tests every cell in the population for its ability to undergo “unlimited” division. Only a fraction of seeded cells retains the capacity to produce colonies. So colony-forming assays were performed to determine the effect of Bmi-1-shRNA on the clonogenicity of A549/SPC-A1 cells. As shown in Fig. 2, the results demonstrated that compared with A549/SPC-A1-wt cells—there was decreased colony formation in A549/SPC-A1-shRNA-Bmi-1 cells ($p < 0.05$).

FIG. 1. Bmi-1-shRNA (B lymphoma Mo-MLV insertion region 1–small hairpin RNA) inhibits the growth and viability of A549/SPC-A1 lung adenocarcinoma cells. (A) Growth curves of lung adenocarcinoma cells were analyzed by the methyl thiazolyl tetrazolium assay. Each value is the mean $\pm$ standard deviation (SD) of triplicate cultures. Bmi-1 knockdown cells grew more slowly than wild-type and control cells ($p < 0.05$); (B) Cell viability of lung adenocarcinoma cells, as determined by the trypan blue exclusion assay. There were no differences in cell viability between Bmi-1 knockdown and control cells ($p > 0.05$).

Bmi-1-shRNA suppresses the tumorigenesis of lung adenocarcinoma cells in vivo

Cells were injected subcutaneously into the armpit of nude mice to observe the effect of Bmi-1-shRNA on the tumorigenicity of A549/SPC-A1 cells *in vivo*. On the 28th day, there was a significant reduction in mean tumor weight in Bmi-1-shRNA cells compared with A549/SPC-A1-wt and A549/SPC-A1-ctr

cells ($p < 0.05$, Fig. 3). However, there is no weight loss, loss of appetite and reduced movement etc in mouse with A549/SPC-A1-shRNA-Bmi-1 compared with the other groups.

Bmi-1-shRNA causes cell cycle arrest in the G0/G1 phase

Cell cycle arrest is one of the major causes of cell growth inhibition. Therefore, cell cycle distribution was measured by

FIG. 2. Effect of Bmi-1-shRNA on colony formation of A549/SPC-A1 cells. Cells were seeded at a density of 300 cells per well in 6-well plates. After 10 days, cells were stained with 0.1% crystal violet. (A) Representative photograph of colony-forming assay of lung adenocarcinoma cells; (B) number of colonies formed by A549/SPC-A1-wt, A549/SPC-A1-ctr, and A549/SPC-A1-shRNA-Bmi-1 cells. Each value is the mean $\pm$ SD of triplicate cultures. * $p < 0.05$ compared with A549/SPC-A1-wt and A549/SPC-A1-ctr cells.

FIG. 3. Effect of Bmi-1 knockdown on the tumorigenesis of A549/SPC-A1 cells *in vivo*. Twenty-eight days after cell inoculation in nude mice, tumors were excised and weighed. Tumor weights of mice inoculated with A549-wt, A549-ctr, or A549/SPC-A1-shRNA-Bmi-1 cells are shown. A549/SPC-A1-shRNA-Bmi-1 cells formed smaller tumors than A549/SPC-A1-wt and A549/SPC-A1-ctr cells. * $p < 0.05$.

FIG. 4. Flow cytometric analysis of the cell cycle in A549/SPC-A1 cells. The cells were harvested and stained with propidium iodide for cell cycle analysis. DNA frequency distribution histograms of A549/SPC-A1-wt, A549/SPC-A1-ctr, and A549/SPC-A1-shRNA-Bmi-1 cells in one representative sample.

flow cytometry to determine whether inhibition of cell growth was associated with cell cycle arrest. As shown in Fig. 4, analysis of cell-cycle distribution showed an accumulation of cells in the G₀/G₁ phase in Bmi-1 knockdown cells compared with wild-type and control cells, with a concomitant decrease in the S phase ($p < 0.05$).

Bmi-1-shRNA downregulates cyclin D1 and upregulates p21 and p27 expression

Cell cycle control is one of the major regulatory mechanisms of cell growth. p53 has been reported to play an important role in G₀/G₁ arrest (Agarwal et al., 1998). However, we found no change in p53 expression in A549/SPC-A1-wt, A549/SPC-A1-ctr, and A549/SPCA1-shRNA-Bmi-1 cells. G₁ phase arrest mainly results from deregulation of cyclin D1 expression (Tashiro et al., 2007); thus, to investigate the signaling pathway underlying the G₁ phase arrest of Bmi-1-shRNA cells, the expression of cyclin D1 protein was determined by western blotting. The results showed that silencing of Bmi-1 expression resulted in reduction of cyclin D1 expression. Some studies have shown that p16, p21, and p27 directly act on the regulation of the G₁/S phase checkpoint (Vidal et al., 2000; Abbas and Dutta, 2009). Since the p16INK4a gene is deleted in A549 cells, only p21 and p27 expression was analyzed by western blotting. The results showed that both p21 and p27 expression increased in A549-shRNA-Bmi-1 cells, and increased in SPC-A1 cells, which have an INK4a locus, although p16 levels remained unchanged (Fig. 5).

Discussion

Here, we showed that Bmi-1 knockdown suppressed the proliferation of lung adenocarcinoma cells *in vitro* and *in vivo*. In general, growth inhibition results from necrosis, apoptosis, or cell cycle arrest. To investigate whether this effect was related to necrosis induction, the trypan blue exclusion test was performed, which showed no significant changes between Bmi-1 knockdown cells and control cells. Therefore, Bmi-1-shRNA did not lead to increased cell necrosis. The cell cycle is a crucial regulator of cell proliferation, which is divided into four phases: G₁, S, G₂, and M. Abnormal cell cycle regulation is an important mechanism of tumorigenesis. The effect of Bmi-1 gene silencing on the cell cycle varies between different cell types. Bmi-1^{-/-} leukemic cells displayed accumulation in the G₁ phase, and fewer cells in the S phase (Lessard, 2003). However, there have also been conflicting reports, as Cui et al. demonstrated that Bmi-1 knockdown by siRNA had no significant effect on the cell cycle in human neuroblastoma 1 BE (2)-C cells (Cui et al., 2006).

In the present study, we used flow cytometry to determine the effect of Bmi-1-shRNA on the cell cycle. The results showed that Bmi-1-shRNA led to inhibition of lung adenocarcinoma cell growth by arresting the cell cycle in the G₀/G₁ phase, accompanied by a reduction of cells in the S phase. The progression of the cell cycle is regulated by many factors, including cyclins, CDKs, and cyclin-dependent kinase inhibitors (Malumbres et al., 2009). Researchers have shown that oncogenes or suppressor genes are linked to cell cycle changes in more than 90% of human tumors, and changes in

FIG. 5. Effects of Bmi-1-shRNA on p16, p27, p21, and cyclin D1 levels of A549/SPC-A1 cells. (A) Protein expression levels of on Bmi-1, p16, p27, p21, and cyclin D1 as determined by western blot analysis; β -actin was used as a loading control. Triplicate experiments showed consistent results. (B) Relative protein expression levels from (A) of Bmi-1, p16, p27, p21, and cyclin D1 compared with β -actin were determined using LabWorks Software. * $p < 0.05$.

G1/S-phase transition genes are the most common (Tashiro et al., 2007).

As a member of the cyclin family, cyclin D1 plays distinct roles in cell cycle progression through the G1 phase. Cyclin D1 binds to CDK4 and CDK6 to form retinoblastoma protein (pRB) kinase, resulting in the inability of pRB to repress the E2F transcription factor. The E2F transcription factor activates the transcription of several genes required for the transition from the G1 to S phase (Tashiro et al., 2007). In addition, the CIP/KIP (*CDK* interacting protein/Kinase inhibitory protein) family, A cyclin-dependent kinase inhibitor, which includes the genes p21, p27 and p57, can halt cell cycle in G1 phase and exert its inhibitory activities by interacting with a variety of cyclin-CDK complexes.

Here, Bmi-1 knockdown inhibited the expression of cyclin D1 and increased the expression of p21 and p27. This suggests that knockdown of Bmi-1 arrests lung adenocarcinoma cells in the G1 phase, possibly via downregulation of cyclin D1 expression, and upregulation of p21 and p27 expression. Previous studies have shown that the Bmi-1 gene can regulate normal cell proliferation, and Bmi-1^{-/-} leukemic cells display proliferation arrest (Cui et al., 2006). In general, Bmi-1 is thought to act through p16INK4A-dependent pathways to regulate proliferation during cancer progression (Kang et al., 2006; Wu et al., 2011). However, Bmi-1 also promotes Ewing sarcoma tumorigenicity independently of p16INK4a repression (Douglas et al., 2008). Thus, the extent to which overexpression of Bmi-1 contributes to downregulation of p16 in lung carcinoma remains unknown (Breuer RH et al., 2005). In the present study, p16 expression in SPC-A1 cells did not change upon Bmi-1 knockdown. Thus, silencing of Bmi-1 expression did not inhibit cell proliferation through the classical pathway in SPC-A1 cells with an INK4a locus.

PI3K/Akt is critical for cell proliferation. It has been reported that Akt can indirectly stabilize cyclin D1 by inhibiting GSK3 β (Engelman et al., 2006), and Bmi-1 can modulate Akt activity in breast cancer cells in a p16INK4a-independent manner (Guo WJet al., 2007). Akt activation also stimulates tumor cell proliferation through multiple cell cycle regulatory targets. Akt triggers a network that positively regulates G1/S cell cycle progression through inactivation of GSK3 β , leading to increased cyclin D1 levels, and decreased levels of Forkhead family transcription factors and the tumor suppressor tuberlin, resulting in reduction of p27 and p21 (Engelman et al., 2006). As a transcriptional repressor, Bmi-1 is unable to directly activate the Akt pathway, while the phosphatase and tensin homolog (PTEN) tumor suppressor negatively regulates the Akt pathway and its downstream target, Bmi-1 (Blanco-Aparicio et al., 2007; Song LBet al., 2009). In our previous study, we found that knocking down endogenous Bmi-1 resulted in reduction of the phosphorylated Akt (p-Akt) levels, and increased expression of PTEN (Meng et al., 2012). Therefore, Bmi-1-shRNA-mediated modulation of cyclin D1, p21, or p27 might occur through the PTEN/Akt pathway in lung adenocarcinoma.

Taken together, our study demonstrates that shRNA-mediated knockdown of Bmi-1 effectively inhibits the proliferation of lung adenocarcinoma cells *in vitro* and *in vivo* through G0/G1 phase arrest. We also conclude that the PTEN/Akt/cyclin D1 pathway or p21 and p27 pathways are involved in Bmi-1 modulation of the cell cycle in lung adenocarcinoma cells in the absence or presence of the INK4a/ARF locus, independently of p16INK4a repression.

Author Disclosure Statement

The authors declared no competing financial interests exist.

Acknowledgments

This work was supported by a grant from Liaoning Province Technology Department of China (grant no. 20072169).

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Received for publication September 18, 2013; accepted after revision December 24, 2013.