Expression of Bmi-1 gene in esophageal carcinoma cell EC9706 and its effect on cell cycle, apoptosis and migration

Ju-Feng Wang, Ying Liu, Wen-Jing Liu, Su-Ying He
Department of Oncology, Henan Tumor Hospital, Zhengzhou, Henan 450003, P. R. China

[Abstract] Background and Objective: Previous studies have shown that Bmi-1 is overexpressed in a variety of tumors, suggesting that Bmi-1 plays an important role in tumorigenesis. In this study, we investigated the effect of Bmi-1 siRNA on cell proliferation, cell cycle, cell apoptosis and migration of human esophageal carcinoma EC9706 cells, and explored its potential mechanisms. Methods: Bmi-1 small interfering RNA (siRNA) was transferred into EC9706 cells. Then, cell proliferation was measured using cell counting kit-8 (CCK-8), cell cycle and cell apoptosis were analyzed by flow cytometry, cell migration ability was detected using Boyden chamber assay, and the mRNA and protein expression levels of Bmi-1, p16, Bcl-2, Bax, and MMP-2 were determined using real-time polymerase chain reaction (PCR) and Western blot analysis, respectively. Results: Bmi-1 siRNA treatment significantly inhibited the expression of Bmi-1 at both mRNA and protein levels in EC9706 cells. Cell proliferation rate decreased dramatically in the Bmi-1 siRNA treated group than in the untreated group and in the scrambled siRNA treated group (both P < 0.001). In Bmi-1 treated group, the percentage of cells at G0/G1 stage was 71.93%, which was higher than that in the untreated group (47.36%) or scramble siRNA treated group (48.47%) (both P < 0.001). Early cell apoptosis rate also increased significantly in the Bmi-1 siRNA treated group (both 17.32%) than in the untreated group (2.61%) and in the scramble siRNA treated group (2.73%) (both P < 0.001). Further experiment suggested that downregulation of Bmi-1 led to less cell migration. In EC9706 cells transfected by Bmi-1 siRNA, the expression levels of p16 and Bax increased, while the expression level of Bcl-2 decreased. Conclusions: Bmi-1 downregulation in esophageal carcinoma cells inhibits cell proliferation, cell cycle, and cell migration, while increases cell apoptosis. These results suggest that Bmi-1 is a potential molecular target of treating esophageal cancer.

Key words: Bmi-1, esophageal carcinoma, cell line, EC9706 cells, proliferation, apoptosis, migration
using real-time polymerase chain reaction (PCR) and Western blot analysis respectively. Our study provides a basis for the treatment of esophageal cancer at the molecular level.

Materials and Methods

Materials

Esophageal carcinoma cell line EC9706 was reserved in our lab. Bmi-1 small interfering RNA (siRNA) (h) and antibodies against Bmi-1, p16, Bcl-2, Bax, MMP-2 and β-actin were purchased from Santa Cruz. One step real-time PCR kit (Quant) was purchased from Tiangen Biotech (Beijing). Cell Counting Kit-8 (CCK-8) was purchased from China Beyotime institute of Biotechnology. Protein lysis buffer was purchased from TaKaRa Biotechnology (Dalian). Boyden chambers were purchased from Qilin Medical Instrument Factory (Haimen, Jiangsu province). Primers for real-time PCR are shown in Table 1. The primers were synthesized by Sangon Biotech (Shanghai).

![Table 1 Primers used for real-time PCR](chart)

<table>
<thead>
<tr>
<th>Prime</th>
<th>Prime sequence (5′→3′)</th>
<th>GenBank ID</th>
<th>Anealing temperature</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bmi-1</td>
<td>Forward, GCCAACAGCAGCCAGGAGG; reverse, ATCTGGGCTTACGCTGAGGCC</td>
<td>NM_005180</td>
<td>54°C</td>
<td>145bp</td>
</tr>
<tr>
<td></td>
<td>p16</td>
<td>GU086367</td>
<td>55°C</td>
<td>196bp</td>
</tr>
<tr>
<td></td>
<td>bcl-2</td>
<td>NM_000633</td>
<td>53°C</td>
<td>150bp</td>
</tr>
<tr>
<td></td>
<td>bax</td>
<td>NM_138763</td>
<td>56°C</td>
<td>201bp</td>
</tr>
<tr>
<td></td>
<td>β-actin</td>
<td>NM_001101</td>
<td>55°C</td>
<td>147bp</td>
</tr>
</tbody>
</table>

Methods

Cell culture and transfection  EC9706 cells were cultured in RPMI-1640 medium containing 100 units/mL penicillin, 100 μg/mL streptomycin, 50 μg/mL azithromycin and 10% FBS at 37°C in a humid incubator with 5% CO₂. Experimental cells were at logarithmic phase. When cells reached more than 90% confluence, EC9706 cells were transfected with either scramble siRNA or Bmi-1 siRNA using Lipofectamine™ 2000 according to the manufacturer’s instruction. Three groups were defined as follows: the untreated group, the scramble siRNA treated group, and the Bmi-1 siRNA treated group.

Cell proliferation assay  CCK-8 is a sensitive, non-radioactive colorimetric analysis, which detects the number of living cells as a means to measure cell proliferation. CCK-8 solution can be directly added to cell medium. Detailed procedures to measure cell growth rates were as follows: cells were harvested 0 h, 24 h, 48 h, 72 h, and 96 h after transfection and then incubated with fresh medium containing 10% of CCK-8 at 37°C for 1–4 h. The absorbance at 450 nm was measured using a microplate reader.

Cell cycle analysis  A total of 1 × 10⁶ untreated and transfected EC9706 cells were harvested 24 h after transfection, rinsed with PBS buffer, and fixed at 4°C for 30 min using 70% cold ethanol, respectively. After fixation, the cells were washed for three times with cold PBS, resuspended in PBS containing 40 μg propidium iodide and 100 μg RNase A and then incubated at 37°C for 30 min. Finally, DNA contents were analyzed by flow cytometry (FCM).

Apoptosis detection by Annexin V-FITC  Apoptosis was determined as described by Lu et al. Apoptotic cells were separated from the untreated group, the scramble siRNA treated group, and the Bmi-1 siRNA treated group.

Cancer cell migration assay  Cell migration was measured using Boyden chamber assay. Briefly, three groups of cells (about 1 × 10⁵ cells in each group) were suspended in 800 μL of cell medium containing 0.2% calf serum, inoculated at the upper Boyden chamber, and cultured for 6 h. Cells which have migrated to the lower membrane were fixed with methanol and stained by HE staining. The invading cells in 30 microscopic views were counted in the lower chamber under 200 x magnifications.

Real-time PCR  Total RNAs were extracted using Trizol reagent. Real-time PCR was then performed using Quant One-step real-time PCR kit according to the manufacturer’s
protocol. Briefly, after the reverse transcription reaction at 50°C for 30 min, cDNAs were initially denatured at 94°C for 2 min, and followed by 35 cycles of repeated procedure as follows: denatured at 94°C for 30 s, annealed for 30 s (the respective annealing temperature for each gene is listed in Table 1), and extent at 65°C for 30 s. PCR was carried out using Applied Biosystems 7300 real-time PCR system. Independent experiments were repeated 3 times for each sample and the relative expression levels of genes were analyzed using $2^{- \Delta \Delta C_{t}}$ method [6].

**Western blot analysis** EC9706 cells were collected in lysis buffer 24 h after transfection and centrifuged down at 16 000 × g for 5 min. The protein concentration was determined using Bradford method. A total of 80 μg proteins were boiled for 5 min, separated in 10% SDS-PAGE, and transferred to a nitrocellulose membrane. The membrane was blocked in TBST containing 5% milk at room temperature for 2 h, and incubated overnight with the primary antibodies in the blocking buffer at 4°C. Subsequently, the membrane was washed with TBST for 3 times and incubated with appropriate secondary antibodies for another 1 h. After washing with TBST for 3 times, the films were developed. The relative protein expression level was quantified using Image-Pro Plus 5.0 software.

**Statistical analyses** SPSS13.0 statistical software was used for statistical analyses. Statistical data were represented as mean ± standard deviation. Student t test was used to compare two samples and the single-factor analysis of variance (One-way ANOVA) was used to compare multiple samples. A value of $P < 0.05$ was considered as significantly different.

**Results**

**Cell proliferation**

Cells in three groups were harvested at 24 h, 48 h, 72 h, and 96 h after Bmi-1 siRNA treatment. CCK-8 kit results showed the proliferation rate of EC9706 cells was not significantly different between the untreated and scramble siRNA treated groups at each time point ($P = 0.700, 0.123, 0.534, 0.459$, respectively). However, the proliferation rate of EC9706 cells was significantly lower in the Bmi-1 siRNA treated group than in the untreated and scramble siRNA treated groups (both $P < 0.001$) (Figure 1). These results suggest that downregulation of Bmi-1 significantly inhibits the proliferation of EC9706 cells.

**Downregulation of Bmi-1 affects cell cycle distribution in EC9706 cells**

Three groups of cells were collected for cell cycle analysis 24 h after treatment. The results showed that 47.36% of the untreated cells and 48.47% of the scramble siRNA treated cells were at G0/G1 phase, which was significantly lower than that of the Bmi-1 siRNA treated cells (71.93%) ($P < 0.001$). By contrast, the number of cells at S phase was lowest in the Bmi-1 siRNA treated group (Figure 2). These results suggest that Bmi-1 siRNA treatment retains EC9706 cells at G0/G1 phase and blocks DNA synthesis.

**Apoptosis analysis**

Three groups of cells were harvested 24 h after treatment and apoptosis was analyzed using flow cytometry. Early stage of apoptosis in the Bmi-1 siRNA treated group (17.32%) was significantly higher than that in the untreated (2.61%) and scramble siRNA treated (2.73%) groups (both $P < 0.001$). There was no statistical difference between the untreated and scramble siRNA treated groups ($P = 0.852$). In addition, the number of living cells in the untreated or scramble siRNA treated groups were significantly larger...
than that in the Bmi-1 siRNA treated group \((P < 0.001)\) (Figure 3). These results indicate that Bmi-1 siRNA induces cell apoptosis in EC9706 cells.

**Cell migration analysis**

Cells were inoculated in the upper chamber 24 h after treatment. Compared with the untreated \((152.29 \pm 5.92)\) and scramble siRNA treated \((147.63 \pm 6.13)\) groups, the number of invading EC9706 cells decreased significantly after Bmi-1 siRNA treatment \((70.38 \pm 5.17)\) \((P < 0.001)\). In addition, no difference between the untreated and scramble siRNA treated groups was observed \((P = 0.360)\). These results suggest Bmi-1 siRNA treatment inhibits the migration of EC9706 cells.

**Gene expression levels**

Total RNAs were extracted 24 h after that cells were treated with scramble siRNA or Bmi-1 siRNA. Real-time PCR were performed using Quant one-step real-time PCR kit. Specific bands were obtained for cell proliferation and cell cycle-related gene p16, apoptosis related genes Bcl-2 and Bax, as well as invasion and metastasis related factor MMP-2 (Figure 4). The mRNA expression levels of these genes were similar in the untreated and scramble siRNA treated EC9706 cells. In the Bmi-1 siRNA treated EC9706 cells, the mRNA expression of Bmi-1, Bcl-2 and MMP-2 was significantly downregulated \((all \ P < 0.001)\), whereas that of p16 and pro-apoptotic gene Bax was upregulated \((both \ P < 0.001)\) (Figure 5).

**Protein expression levels**

Total proteins were extracted 24 h after treatment and were subjected to Western blot analysis. There was no difference in the expression levels of Bmi-1, p16, Bcl-2, Bax and MMP-2 between the untreated and scramble siRNA,
treated groups \( (P = 0.159, 0.204, 0.211, 0.093, 0.051, \) respectively). However, the expression level of Bmi-1, Bcl-2, and MMP-2 decreased significantly in the Bmi-2 siRNA treated groups \( (all \ P < 0.001) , \) while that of p16 and Bax increased significantly \( (both \ P < 0.001) \) (Figure 6).

**Discussion**

Bmi-1 was first characterized in cancer cells by the Netherlands Cancer Institute in 1991, which is involved in the proliferation of cancer stem cells and tumorigenesis\(^7\). Increasing evidence showed that Bmi-1 not only plays a critical role in tumorigenesis and tumor growth, but is also a valuable factor in clinical prognosis\(^\text{8,9} \). Thus, Bmi-1 is a new potential marker for cancer diagnostic and therapeutic target. Studies have shown that the expression level of Bmi-1 increases in a variety of malignant tumors, and is related to prognosis of many cancers such as gastric cancer\(^2\), ovarian cancer\(^\text{9,10} \), chronic myeloid leukemia\(^\text{108} \), head and neck cancer\(^8 \), breast\(^\text{9} \), bladder cancer\(^\text{11} \), and so on. However, the role of Bmi-1 in esophageal cancer is unknown yet. In this study, we transfected Bmi-1 siRNA into EC9706 cells to study the effect of Bmi-1 downregulation on cell proliferation, cell cycle, cell apoptosis and invasiveness, and explored its mechanisms. The present study aimed to provide evidence of this new therapeutic target for treating esophageal cancer.

---

**Figure 3**  The result of cell apoptosis in three group cells

A, effect of Bmi-1 siRNA on cell apoptosis of EC9706 cells. B, columns, means of three independent experiments; bars, SD.

* \( P < 0.05 \), compared with untreated group and control siRNA group. I, percentage of viable cells; II, percentage of early apoptotic cells; III, percentage of late apoptotic and dead cells; IV, percentage of necrotic cells.
Recent studies showed that Bmi-1 plays an important role in the regulation of tumor cell proliferation and cell cycle. Knockout of Bmi-1 gene in human hepatocarcinoma HepG2 cells inhibits cell proliferation. Jacobs et al. studied the function of Bmi-1 in cell proliferation using mouse embryonic fibroblasts (MEFs). They found that cells are difficult to enter S phase when Bmi-1 is repressed. The proliferation rate and cell density of wild type MEFs are significantly higher than those of the Bmi-1 knockout ones. In addition, knockout of Bmi-1 gene in gastric cancer cell
AGS leads to inhibition of cell growth\[2\]. In this study, we found that downregulation of Bmi-1 using Bmi-1 siRNA inhibits the proliferation of esophageal carcinoma EC9706 cells. A majority of cells are arrested at G0/G1 phase and DNA synthesis is inhibited, which eventually leads to the inhibition of proliferation. P16 was found as a new anti-cancer gene by Kamb et al. in Cold Spring Harbor Laboratory in 1994. P16 is an essential cell cycle related gene and participates in cell cycle regulation. P16 inhibits cell cycle-dependent kinase 4 (CDK4) directly, and thereby inhibits cell cycle progression and cancer cell growth. To identify whether p16 is involved in Bmi-1 regulation of cancer cell growth, we detected the expression levels of p16. We found that the expression of p16 increases significantly at both mRNA and protein levels. These data suggest that the inhibition of EC9706 cell proliferation and cell cycle progression by Bmi-1 downregulation is mediated by the upregulation of p16.

Tumorigenesis is the result of an imbalance between cell proliferation and cell apoptosis. It is an effective way to treat cancer by inducing cancer cell apoptosis. Some studies have shown that the effect of Bmi-1 downregulation on cell cycle and cell apoptosis is cell-type specific. The proportion of Bmi-1-/- leukemia cells at G0/G1 phase increases than that of wild-type cells. In the meanwhile, the proportion of cells at S phase decreased and cell apoptosis increases\[24\]. However, neither cell cycle nor apoptosis is affected in human neuroblastoma BE (2)-C cells by the downregulation of Bmi-1\[15\]. Our results showed that the early apoptosis rate of EC9706 cells treated with Bmi-1 siRNA is significantly higher than that of the untreated and scramble siRNA treated cells. Because anti-apoptotic gene Bcl-2 and pro-apoptotic gene Bax are two important regulators of cell apoptosis, we further detected the expression levels of these two genes at mRNA and protein levels using real-time PCR and Western blot. We found that the expression level of Bcl-2 is significantly lower in the Bmi-1 siRNA treated group than in the untreated and scramble siRNA treated groups, while the expression level of Bax is higher. Therefore, inhibition of Bmi-1 expression induces cell apoptosis, which may be mediated by the downregulation of Bcl-2 and upregulation of Bax. Further studies are needed to delineate the additional mechanisms.

In addition to participating in malignant tumorigenesis and tumor progression, Bmi-1 is implicated in tumor invasion, metastasis, and prognosis. By analyzing transgenic mice and 1153 patients who were diagnosed with ten different types of cancer using comparative genomics approach, Glinsky et al.\[16\] suggested 11 genes exists in the conservative Bmi-1 signaling pathway. Further studies showed that the expression patterns of these 11 genes may predict tumor metastasis, recurrence, and death in various
types of cancer patients. In addition, the activation of Bmi-1 associated PcG pathway plays an important role in the progression of metastatic prostate cancer\cite{17}. Feng et al.\cite{18} found that Bmi-1 overexpression is co-related with breast cancer metastasis and TNM stage. In this study, we detected migration of EC9706 cells after Bmi-1 siRNA treatment using Boyden chamber assay and measured the expression of a closely related factor, MMP-2, using real-time PCR and Western blot. Our results showed that Bmi-1 siRNA decreases significantly EC9706 cell migration. In addition, the expression of MMP-2 at both mRNA and protein levels decreases significantly. These results imply a direct relationship between lower EC9706 cell migration and lower expression of MMP-2, however, specific mechanism remains to be elucidated.

In summary, this study focuses on the role of Bmi-1 in esophageal cancer, and reveals that the inhibition of Bmi-1 expression inhibits cell growth, arrests cell cycle, induces cell apoptosis, and decreases cell invasion, which are closely related to the changes in the expression of p16, Bcl-2, Bax, and MMP-2. Further studies illuminating the molecular mechanisms of cancer inhibition by Bmi-1 will shed light on using Bmi-1 as a molecular target to treat esophageal cancer.

References


