Silencing Bmi-1 enhances the senescence and decreases the metastasis of human gastric cancer cells

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AIM: To evaluate the impact of Bmi-1 on cell senescence and metastasis of human gastric cancer cell line BGC823.

METHODS: Two pairs of complementary small hairpin RNA (shRNA) oligonucleotides targeting the Bmi-1 gene were designed, synthesized, annealed and cloned into the pRNAT-U6.2 vector. After DNA sequencing to verify the correct insertion of the shRNA sequences, the recombinant plasmids were transfected into BGC823 cells. The expression of Bmi-1 mRNA and protein was examined by reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting. The effects of Bmi-1 knockdown on cell senescence and metastasis were determined by the β-Gal activity assay and Boyden chamber assay, respectively.

RESULTS: The double-stranded oligonucleotide fragments of Bmi-1 short interfering RNA (siRNA) cloned into pRNAT-U6.2 vector conformed to the inserted sequence. RT-PCR and Western blotting indicated that the expression levels of Bmi-1 gene mRNA and protein were markedly decreased in transfected BGC823 cells with pRNAT-U6.2-si1104 and pRNAT-U6.2-si1356, especially in transfected BGC823 cells with pRNAT-U6.2-si1104, compared with two control groups (empty vector and blank group). In particular, Bmi-1 protein expression was almost completely abolished in cells transfected with the recombinant vector harboring shRNA targeting the sequence GGAGGAGGTGAATGATAAA (nt1104-1122). Compared with untransfected cells and cells transfected with the empty vector, the mean percentage of senescent cells increased and the number of cells passing through the Matrigel decreased in cells transfected with the recombinant vectors.

CONCLUSION: Silencing Bmi-1 by RNA interference can increase the senescent cell rate and effectively reduce the metastasis of gastric cancer cells.

Key words: Bmi-1; Gastric cancer; Senescence; Metastasis

Core tip: The overexpression of Bmi-1 contributes to the development of cancers. This study aimed at to evaluate the impact of Bmi-1 on the senescence and metastasis of human gastric cancer. The results demonstrated that inhibition of Bmi-1 gene expression can enhance the senescence of human gastric cancer cells and inhibit the invasion and metastasis of gastric cancer. This research has provided an indication that Bmi-1 inhibitors might be developed as new agents for gastric cancer.
INTRODUCTION

Bmi-1 (B lymphoma-Mo-MLV insertion region 1 homolog), a member of the polycomb group (PcG), functions as a transcriptional repressor and presents with high expression in many tumors, indicating a poor prognosis[1,2]. Several lines of evidence suggest that Bmi-1 blocks cell senescence and proliferation[3,4], and the Bmi-1 gene is also associated with tumor invasion and metastasis[5]. Based on a list of genes on a wild-type and Bmi-1-deficient genetic background, Bmi-1 has been identified as a predictor of the response to therapy and survival in multiple types of cancer[6,7]. Therefore, this study intended to silence Bmi-1 in BGC823 cells by RNA interference, to observe the role of Bmi-1 in the senescence and metastasis of gastric cancer cells.

MATERIALS AND METHODS

Materials

Short interfering RNA (siRNA) vector pRNAT-U6.2 was purchased from GenScript Inc. (Piscataway, NJ, United States), Bmi-1 antibody from Santa Cruz Biotechnology (CA, United States). Bgl II, Hind III and T4DNA ligase were obtained from Promega. BGC823 human gastric cancer cell lines were received from the Chinese Academy of Science. RPMI 1640 and fetal bovine serum were supplied by Gibco BRL (Grand Island, NY, United States). Liposomes LipofectAMINE™-2000, G418, Trizol reagent and reverse transcription-polymerase chain reaction (RT-PCR) kit were purchased from Invitrogen (Carlsbad, CA, United States) and senescence β-galactosidase staining kit (Cell Signaling Technology, Beverly, MA, United States).

Methods

Selection of siRNA for Bmi-1 target sequence: The analysis and design of Promega siRNA target sequence scanned human Bmi-1 gene sequence (NM_005180) was based on the design principle of siRNA target sequence. The 19bp siRNA target sequences, including 1104nt-1122nt (GGAGAGGTGACTGACATGACCAAAATTTCGAAATTTTCAAGAAGATGGAAGTTGAGGAGGTGAATGATATGACCGACAGGAGGGGAGGTGAATGATAATTCAAGAGATTTATCATTCACCTCCTCCTTTTTTC-3’, 1104F: 5’-GATCCGGAGGAGGTGAATGATAATTCAAGAGATTTATCATTCACCTCCTCCTTTTTTC-3’, 1104R: 5’-TCGAGAAAGGAGGAAGTTGAAATTTTCAAGAAGATGGAAGTTGAGGAGGTGAATGATATGACCGACAGGAGGGGAGGTGAATGATAATTCAAGAGATTTATCATTCACCTCCTCCTTTTTTC-3’), and 1356nt-1374nt (GAGAGATGGACTGACAAATTTCAAGAGATTTATCATTCACCTCCTCCTTTTTTC-3’, 1356F: 5’-GATCCGGAGGAGGTGAATGATAATTCAAGAGATTTATCATTCACCTCCTCCTTTTTTC-3’, 1356R: 5’-TCGAGAAAGGAGGAAGTTGAAATTTTCAAGAAGATGGAAGTTGAGGAGGTGAATGATATGACCGACAGGAGGGGAGGTGAATGATAATTCAAGAGATTTATCATTCACCTCCTCCTTTTTTC-3’), were synthesized (1104F and 1104R, 1356F and 1356R), as the target sequence after the BLAST homology analysis and design of Promega siRNA target sequence. Selection of siRNA for Bmi-1 target sequence: The single-stranded DNA oligonucleotide (1104F and 1104R, 1356F and 1356R) was converted into a double-stranded DNA (si1104 and si1356) by conventional annealing, and reconnected overnight at 4 °C, utilizing 2 × reaction reconnected buffer (5 μL), linear pRNA-U6.2 vector (1 μL), T4 ligase (1 μL) and annealing product (3 μL). The two recovered products were incubated at 16 °C for 16 h after addition of Solution I containing DNA ligase, and the resulting ligated products were used to transfect well-prepared competent E. coli DH5α. The whole transfection mix was plated onto a prewarmed LB-ampicillin (AMP) agar plate and then incubated at 37 °C for 12 h. Individual growing colonies were picked out and incubated at 37 °C for 12 h in LB broth containing AMP. Full length plasmid DNA was extracted from positive clones using a plasmid DNA extraction kit and then subject to testing for the presence of Bmi-1 with nuclease digestion using Bgl II, Hind III and T4DNA ligase.

Identification of recombinants: The recombinants were identified by PCR amplification, using primers PRNA-U6.2 FORWARD and PRNA-U6.2 REVERSE. PCR reaction was performed with 3 min of initial denaturation at 94 °C, 35 cycles of 45 s denaturation at 94 °C, 45 s annealing at 55 °C, 45 s extension at 72 °C, and finally 10 min extension at 68 °C. RT-PCR amplification products were electrophoresed and inspected on a 1.1% agarose gel, and recovered and purified by using DNA Gel recovery kit.

Transfection by liposome-mediated siRNA: The transfection process was according to the Lipofectamine™ 2000 instructions: a cell suspension containing 4-8 × 10^6 cells was added to 500 μL of growth medium with serum but without antibiotics; 0.8-1.2 μg DNA was added to 50 μL of medium without serum; 2 μL of Lipofectamine™ 2000 was added to 50 μL OptiMEM® 1 medium and incubated for 5 min at room temperature; the DNA-Lipofectamine™ 2000 complexes were added and incubated for 4 h at 37 °C in a CO2 incubator. Finally cells were assayed at 24-48 h post-transfection for the appropriate activity.

RT-PCR analysis: RT-PCR was carried out as described previously[8]. Cells were harvested and rinsed with phosphate-buffered saline (PBS) at corresponding time points and total RNA in the treated sections was extracted according to the total RNA extracting kit. A solution was added consisting of 10 mmol/L dNTP, 0.5 g/L oligo(dT), 40 U reverse transcriptase (m-mulv), 59 pH 8.3 RT buffer (250 mmol/L Tris-HCl, 250 mmol/L KCl, 20 mmol/L MgCl2, 50 mmol DTT) and deionized...
Figure 1: Annealing of siRNA hairpin DNA by electrophoresis. M: DNA marker; 1: Hairpin single-stranded DNA products for 1104F and 1104R; 2: Hairpin single-stranded DNA product for 1356F and 1356R.

RESULTS

Analysis of siRNA hairpin DNA

After annealing of hairpin single-stranded DNA for 1104 and 1356, the electrophoresis showed bright bands below 100 bp, consistent with the design (Figure 1).

Identification of Bmi-1 siRNA vectors

Two hairpin single-stranded DNA products (si1104 and si1356) were connected with pRNAT-U6.2 plasmid to transfect well-prepared competent E. coli DH5α. More than 10 transfected colonies grew on the Amp + LB culture plate. Ten transfected colonies were randomly selected. The DNA sequence of the inserted fragments was consistent with the designed positive recombinants (pRNAT-U6.2-si1104 and pRNAT-U6.2-si1356) (Figure 2).

Expression of Bmi-1 mRNA

The expression of Bmi-1 mRNA was inhibited in transfected BGC823 cells with pRNAT-U6.2-si1104 and pRNAT-U6.2-si1356, especially in pRNAT-U6.2-si1104 transfected BGC823 cells, while two control groups (empty vector and blank groups) had significantly higher levels of Bmi-1 mRNA (P < 0.01) (Figure 3A).
Expression of Bmi-1 protein
There were high levels of Bmi-1 protein by Western blotting in non-transfected and transfected BGC823 cells with empty vector pRNAT-U6.2, compared with transfected BGC823 cells targeting Bmi-1 (pRNAT-U6.2-si1104 and pRNAT-U6.2-si1356). while there was no Bmi-1 expression in the transfected BGC823 cells with pRNAT-U6.2-si1104 targeting Bmi-1 (P < 0.01) (Figure 3B).

Silencing Bmi-1 increased the senescent cell rate and reduced the metastasis of BGC823 cells
The senescent rate of transfected BGC823 cells with pRNAT-U6.2-si1104 and pRNAT-U6.2-si1356 significantly increased compared with the non-transfected and transfected BGC823 cells with empty vector pRNAT-U6.2 (P < 0.01). The number of transfected BGC823 cells with pRNAT-U6.2-si1104 and pRNAT-U6.2-si1356 through the Matrigel significantly decreased, compared with the non-transfected and transfected BGC823 cells with empty vector pRNAT-U6.2 (P < 0.01) (Table 1).

**DISCUSSION**
This study aimed to investigate the impact of Bmi-1 on the senescence and metastasis of human gastric cancer cells, and our results indicate that inhibition of Bmi-1 gene expression can enhance the senescence of human gastric cancer cells and limit the invasion and metastasis of human gastric cancer cells.

Gastric cancer, the most common gastrointestinal malignancy, is the fourth most commonly diagnosed malignancy and the second leading cause of cancer-related death in the world. Gastric cancer is often either asymptomatic or has nonspecific symptoms in its early stages.
Once symptoms become apparent, the cancer has often reached an advanced stage and may also have metastasized and spread to other parts of the body. Accordingly, gastric cancer has a relatively poor prognosis since invasion and metastasis are important prognostic factors\(^{[10,11]}\). Currently, there is evidence that the incidence of gastric cancer is related to multiple oncogenes, such as \(C-myc\), \(Ras\), \(Hst\) and \(C-erbB-2\)^{[12-14]}. The \(Bmi-1\) gene, a polycomb gene (PcG), has been reported as an oncogene with high expression in cancers, and this may be related to high aggressiveness, such that overexpression of \(Bmi-1\) is associated with poor prognosis\(^{[1,7]}\). Compelling research has supported that the expression of \(Bmi-1\) decreases tumor cell senescence and proliferation, and increases tumor invasion and metastasis. The \(Bmi-1\) gene can be synergistic with \(C-myc\) to induce cell metastasis and tumor formation\(^{[13,14]}\). This study demonstrated that the inhibition of \(Bmi-1\) gene expression can increase the senescence of gastric cancer cells and slow down the invasion and metastasis of gastric cancer cells. It has provided further evidence of a role for \(Bmi-1\) in the pathogenesis of gastric cancer.

The senescence β-galactosidase staining kit is designed to detect β-galactosidase activity at pH 6, a known characteristic of senescent cells not found in presenescent, quiescent or immortal cells\(^{[17,18]}\). Boyden chamber assays are used to measure cell invasion and various types of cell migration\(^{[19,20]}\). In this study, the incidence of senescent gastric cancer cells was most obvious when \(Bmi-1\) expression was inhibited, according to β-galactosidase activity. Meanwhile, the number of gastric cancer cells through the Matrigel significantly decreased after inhibiting \(Bmi-1\) expression in the Boyden chamber assay, indicating that the inhibition of \(Bmi-1\) expression can limit the invasion and metastasis of gastric cancer cells. These results suggest that inhibition of \(Bmi-1\) gene expression can enhance cell senescence and reduce the capability for cell invasion and metastasis.

In conclusion, we documented in the present study that silencing \(Bmi-1\) by RNA interference enhances the senescent cell rate and effectively reduces the metastasis of gastric cancer cells. Many studies have shown that \(Bmi-1\) is essential in multiple pathways in the pathogenesis of gastric cancer. Other reports have suggested that \(Bmi-1\) inhibitors have therapeutic potential for gastric cancer through various mechanisms. The current has provided additional support for the notion that \(Bmi-1\) inhibitors might be developed as new agents for gastric cancer.

**COMMENTS**

**Background**

\(Bmi-1\) (B lymphoma Mo-MLV insertion region 1 homolog) has been reported as an oncogene that plays an important role in several types of cancer. The amplification and overexpression of \(Bmi-1\) contribute to the development of many tumors and cancers, such as skin, prostate, breast, ovarian, and colorectal, as well as hematological malignancies. Whether \(Bmi-1\) influences cell senescence and metastasis of human gastric cancer remains unknown. The aim of this study was to evaluate the impact of \(Bmi-1\) on cell senescence and metastasis of the human gastric cancer cell line BGC823.

**Research frontiers**

\(Bmi-1\) is essential in multiple pathways in the pathogenesis of gastric cancer. The role of \(Bmi-1\) on cell senescence and metastasis of human gastric cancer remains unclear.

**Innovations and breakthroughs**

The inhibition of \(Bmi-1\) gene expression can enhance the senescence of gastric cancer cells and limit the invasion and metastasis of gastric cancer cells.

**Applications**

\(Bmi-1\) inhibitors have therapeutic potential for gastric cancer through various mechanisms. This research has provided additional support for the notion that \(Bmi-1\) inhibitors might be developed as new agents for gastric cancer.

**Peer review**

This study demonstrated that the inhibition of \(Bmi-1\) gene expression can increase gastric cancer cell senescence and inhibit invasive behavior in a well-accepted Boyden chamber model. The present study focused on the role of \(Bmi-1\) in cell senescence and metastasis. It would help to understand the mechanism

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Gao FL et al. Silencing Bmi-1 decreases metastasis of gastric cancer

![Figure 3 Levels of Bmi-1 mRNA and protein](image-url)
of Bmi contribution to cancer progression. The data presented in this manuscript are quite good and very supportive of the hypothesis tested.

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