Bmi-1 siRNA inhibited ovarian cancer cell line growth and decreased telomerase activity

T. XIN*, F. B. ZHANG†, G. J. SUI† and X. M. JIN‡

*Second Affiliated Hospital of Harbin Medical University; †Affiliated Oncology Hospital of Harbin Medical University; and ‡Harbin Medical University, Harbin, Heilongjiang 150086, P.R. China

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ABSTRACT

The knockdown of *Bmi-1* could effectively suppress cancer cell proliferation and tumourigenicity in several cancers. This study aims to investigate whether or not *Bmi-1* plays a causative role in the proliferation of ovarian epithelial cancer cells and telomerase activity. The messenger RNA (mRNA) and protein expression levels of *Bmi-1* in the human ovarian carcinoma cell line OVCAR-3 were down-regulated by *Bmi-1* siRNA, as confirmed by real-time polymerase chain reaction (PCR) and Western blot. Cell viability was analysed by MTT assay, and telomerase activity was analysed by a modified telomeric repeat amplification protocol. Targeting *Bmi-1* with siRNA inhibited *Bmi-1* mRNA over five-fold compared with the control cells, and inhibited Bmi-1 protein expression over three-fold compared with control cells. The viability of the OVCAR-3 ovarian cancer cell line was reduced by *Bmi-1* mRNA compared to control cells. Telomerase activity was decreased 22.73% (from 0.33 to 0.255) by *Bmi-1* siRNA treatment compared to control cells. As *Bmi-1* siRNA depressed telomerase activity, cell immortalisation may be prevented; thus, silencing *Bmi-1* may be a potential therapy to manage ovarian cancer.

KEY WORDS: Cell proliferation.
Ovarian neoplasms.
Telomerase.
Tumorigenicity tests.

Introduction

Most cases of ovarian cancer are diagnosed at an advanced stage, resulting in a very poor prognosis. Furthermore, although ovarian cancer initially responds to chemotherapy, it frequently recurs and progresses after initial response. This clinical barrier to its control indicates the need to develop new therapeutic strategies for ovarian cancer.

Ovarian cancer develops and progresses through a complex process that involves proto-oncogenes being activated and anti-oncogenes being deactivated, absent or mutated. The B-cell-specific murine leukaemia virus insertion site 1 (*Bmi-1*) gene plays an oncogenic role in several types of human cancer.[1,2] As *Bmi-1* is a *c-Myc*-cooperating cellular gene in murine lymphomas and a polycomb group transcription repressor gene, it is expressed during normal replication of primary human cells, and prolongs the cell cycle.[3]

Over-expressed *Bmi-1* has been detected in human cancers,[4] including breast cancer,[4-6] cervical cancer,[7] ovarian cancer,[8] prostate cancer,[9] bladder cancer,[10] lung cancer,[1] head and neck cancer,[11] nasopharyngeal cancer,[12] gastric cancer,[13] pancreatic cancer,[14] and colorectal cancer.[15,16] Also, *Bmi-1* has been implicated in telomerase activation in epithelial cells,[4] where its ability to enhance telomerase activation and over-proliferate serial epithelial cells suggests that it may have another fundamental role in carcinogenesis.

The authors previously reported that the Bmi-1 protein is highly expressed in ovarian epithelial cancer tissues and that elevated expression of Bmi-1 correlated closely with increased telomerase activity.[8] The activation of telomerase is considered to be a critical step in carcinogenesis,[17] as telomerase activation directly correlates to both the immortalisation of cells and to tumourigenesis.[8] Over 85% of malignant tumour tissues have enhanced expression of telomerase.[15]

Silencing *Bmi-1* may contribute to the development of anticancer therapies. When Bmi-1 activity was lost through RNA interfere (RNAi), both the growth and tumourigenicity of neuroblastoma and ovarian adenocarcinoma were effectively suppressed, while normal cells were minimally affected.[18] Owing to the pathological and therapeutic significance of *Bmi-1* in cancer, the authors investigate the ability of miRNA to regulate *Bmi-1* in ovarian cancer cells and to affect telomerase activity. The expression of *Bmi-1* is silenced with miRNA in the OVCAR-3 ovarian carcinoma cell line, and then cell viability and telomerase activity are studied.

Correspondence to: Dr GuangJie Sui
Affiliated Oncology Hospital of Harbin Medical University, Harbin, Heilongjiang 150046, P.R. China
Email: guangjiesui123@hotmail.com

Materials and methods

Materials

The human ovarian carcinoma cell line OVCAR-3 was obtained from Shanghai Institute for Biological Sciences, Chinese Academy of Sciences. The enzymes BamH I and EcoR I, and the DH5α-competent cells, were obtained from Takara (TaKaRa Bio Dalian, China), the UNIQ-10 Column DNA reclaiming kit and the plasmid extract kit from Shanghai Sangon Biological Engineering Technology & Services (Shanghai, P.R. China), the plasmid mini kit from

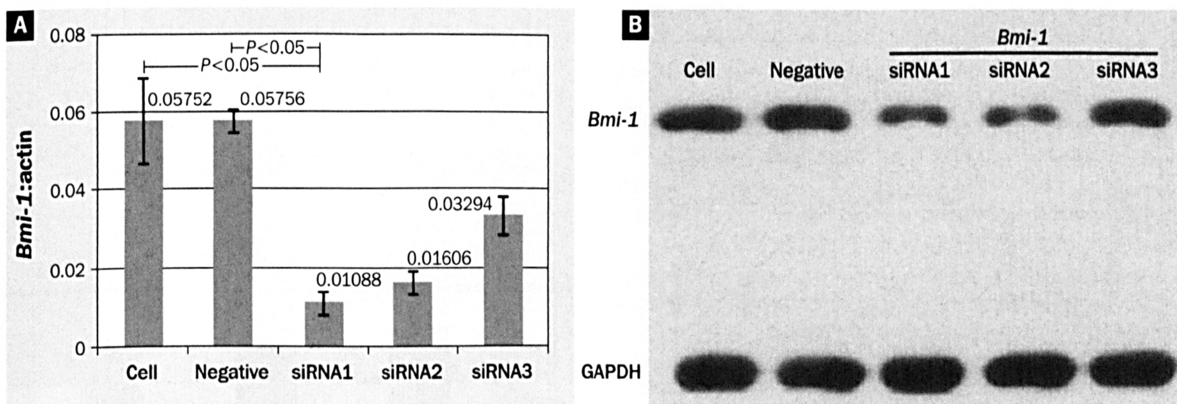

Fig. 1. Silencing *Bmi-1* with siRNA affected expression. **A)** The expression of *Bmi-1* mRNA in OVCAR-3 cells was decreased by treatment with siRNA1 (RNAi-1), siRNA2 (RNAi2), and siRNA3 (RNAi3), compared to untreated cells (Cell) or treated with negative control siRNA (Negative), as measured by RT-PCR. The ratio of *Bmi-1* to actin is illustrated. **B)** The expression of Bmi-1 protein was decreased in cells treated with *Bmi-1* siRNA. The *Bmi-1* protein has a molecular weight of 41 kDa and was compared to that of GAPDH (MW 36 kDa).

Qiagen (Shanghai), Lipofectamine 2000 from Invitrogen (Carlsbad CA, USA), the mouse anti-human Bmi-1 monoclonal antibody from Upstate, New York USA, the ECL kit from Shanghai Pufei Bio-Technology (Shanghai, P.R. China) and the TRAP-Hyb kit from Wuhan Uscn Sciences (Wuhan, P.R. China).

Construction of siRNA expression plasmid

The authors designed three *Bmi-1* siRNA sequences and a negative control sequence using software: siRNA1, 5′-GTT CACAAGACCAGACCAC-3′; siRNA2, 5′-GATAGAGGAGA GGTTGCAG-3′; siRNA3, 5′-GATCAGTCACCAGAGAGAT-3′; and negative control, 5′-TGCAGTTCGGAATCAGCTT-3′. For the reverse transcriptase polymerase chain reaction (RT-PCR), a forward and reverse complementary DNA template were designed for each of the three Bmi-1 siRNA sequences and for the negative control: Bmi-1-f1, 5′-GATCCGTTCACA AGACCAGACCACcttcctgtcagaGTGGTCTGGTCTTGTGAAC TTTTTG-3′; Bmi-1-r1, 5′-AATTCAAAAAGTTCACAAGACC AGACCACtctgacaggaagGTGGTCTGGTCTTGTGAACG-3′; Bmi-1-f2, 5′-GATCCGATAGAGGAGAGGTTGCAGcttcctgt cagaCTGCAACCTCTCCTCTATCTTTTTG-3′; Bmi-1-r2, 5′-AATT CAAAAAGATAGAGGAGAGGTTGCAGtctgacaggaag CTGCAACCTCTCCTCTATCG-3′; Bmi-1-f3, 5′-GATCCGATC AGTCACCAGAGAGATcttcctgtcagaATCTCTCTGGTGACTGA TCTTTTTG-3′; Bmi-1-r3, 5′-AATTCAAAAAGATCAGTCAC CAGAGAGATtctgacaggaagATCTCTCTGGTGACTGATCG-3′; negative-f, 5′-GATCCTGCAGTTCGGAATCAGCTTTTCAAG AGAAAGCTGATTCCGAACTGCATTTTTG-3′; and negative-r, 5′-AATTCAAAAATGCAGTTCGGAATCAGCTTTCTCTT GAAAAGCTGATTCCGAACTGCAG-3′. The single-stranded DNA was diluted to 1 μg/μL, renatured to form complementary double-stranded DNA, cut with restriction enzymes, and then linearised to the siRNA plasmid expression vector.

The DH5α-competent cells were transformed with the plasmids. The amplified DNA plasmids were extracted with a Qiagen plasmid mini prep kit and then further verified by sequencing. The purity and concentrations of the plasmids were determined spectrophotometrically by ultraviolet (UV) absorbance at 260 nm and 280 nm.

Cell culture and transient transfection

The OVCAR-3 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). When the density of the attached cells reached 80%, they were transfected with plasmid using the Lipofectamine 2000 transfection kit. The transfection efficiency was detected 48 h after transfection by observing cell density, cell form, and expression of fluorescence.

Real-time PCR

Total RNA were extracted with Trizol (Invitrogen, USA) and then 20 μL total RNA was used to generate complementary DNA (cDNA) with reverse transcriptase M-MLV reagent (TaKaRa, Japan). The RNA concentration was determined by UV spectrophotometry, and the RNA quality was detected by agarose gel electrophoresis. The primers are shown in Table 1.

Western blots

After the cells were transfected for 48 h they were harvested and total protein was isolated and quantified by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to a PVDF membrane, blocked with 5% fat-free milk in Tris-buffered saline (TBS) containing 0.05% Tween-20, incubated with a primary antibody against Bmi-1 (1 in 500 dilution) overnight at 4°C, and then incubated with secondary antibody conjugated to horseradish peroxidase (HRP; 1 in 5000 dilution) for one hour at room temperature. The protein was detected using

Table 1. Primers for *Bmi-1* and β-actin used in RT-PCR.

Gene		Two-way primer sequences	Reannealing temperature (°C)	Product length (bp)
Bmi-1	Forward:	5′-ATTCCCTCCACCTCTTCTTGTT-3′	57	111
	Reverse:	5′-GCTGGGGCTGTTGCTGGTT -3′		
β-actin	Forward:	5′-CTGTACGCCAACACAGTGC-3′	57	211
	Reverse:	5′-ATACTCCTGCTTGCTGATCC-3′		

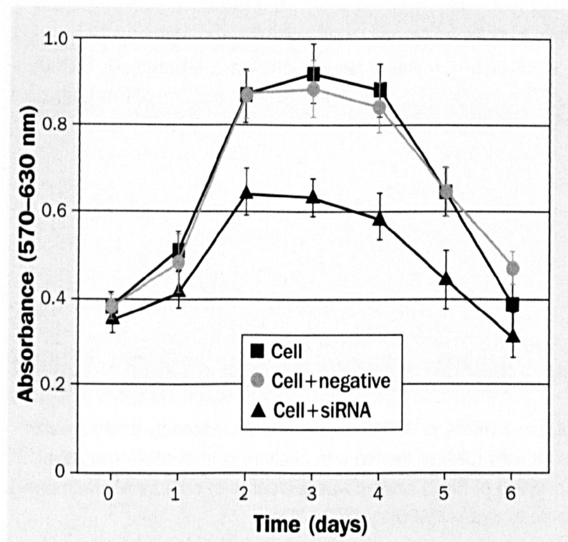

Fig. 2. The MTT assay analysed the cell viability of OVCAR-3 cells treated with *Bmi-1* siRNA. The OVCAR-3 cells were untreated (black squares), treated with negative control siRNA (grey circles), or treated with *Bmi-1* siRNA (black triangles), and their cellular viability was analysed daily with the MTT assay, beginning on the day of the siRNA treatment (day 0).

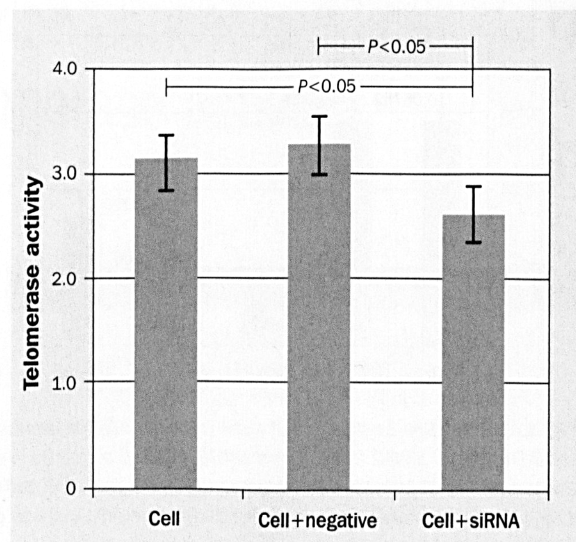

Fig. 3. Telomerase activity of OVCAR-3 cells treated with *Bmi-1* siRNA cells was inhibited. The TRAP assay determined the telomerase activity of cells that were untreated (Cell), treated with negative control siRNA (Cell+negative), and treated with *Bmi-1* siRNA (Cell+siRNA1). Bars indicate standard deviations.

an electrochemiluminescence (ECL) kit (Pierce, USA), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the protein loading control.

Cell viability assay

The MTT assay was performed on untreated OVCAR-3 cells, on OVCAR-3 cells transfected with the plasmid containing the negative control siRNA, and on OVCAR-3 cells transfected with the plasmid containing the siRNA1 DNA. The cells were inoculated on a cell-culture dish (3 cm) and cultivated in DMEM supplemented with 10% FBS medium without antibiotics for 24 h. Then, the cells were transfected using the Lipofectamine 2000 kit. After 24 h, 3×10^4 cells from the three treatments were inoculated into each well of a 96-well plate, where their absorbance (570–630 nm) was detected by spectrophotometry every 24 h, beginning at 0 hour. Viability was plotted as absorbance (570–630 nm) against days after transfection.

Modified telomeric repeat amplification protocol assay for telomerase activity

The modified telomeric repeat amplification protocol (TRAP) assay for telomerase activity used a protocol[19] that was modified for a previous study.[8] When the OVCAR-3 cells were in the exponential phase of growth, they were inoculated into 96-well culture plates in the same treatment groups as those used for the MTT assay. The positive control was a cellular extract from the human embryonic kidney cell line 293, which has constant positive telomerase activity, and the negative control was cell-free lysis buffer.

Statistical analysis

All experiments were performed in triplicate. Statistics were analysed using the SPSS 13.0 software package (Bizinsight [Beijing] Information Technology, Beijing, P.R. China). The ANOVA test was used for comparisons of more than two groups. Results were considered significant at $P<0.05$.

Results

Expression of Bmi-1

To determine the efficiency of the three *Bmi-1* siRNA plasmids, they were compared by RT-PCR after the cells were transfected for 48 h (Fig. 1). The most effective *Bmi-1* siRNA plasmid was found to be RNAi-1, which showed the largest reduction in *Bmi-1* mRNA and protein level. Treating OVCAR-3 cells with RNAi-1 resulted in a *Bmi-1* mRNA:actin mRNA ratio of 0.011, which was over five-fold lower than the ratio of untreated cells or of cells treated with the negative control siRNA ($P<0.05$). The Bmi-1 protein:GAPDH ratio was 0.180 in the cells treated with the RNAi-1 siRNA, which was the lowest of the three *Bmi-1* siRNAs tested, and was over three-fold lower than the untreated cells (0.602) and the ratio of the cells treated with negative control siRNA (0.607) ($P<0.05$). RNAi-1, which was the most efficient in knocking down *Bmi-1*, was used in the following experiments.

Cell viability

To understand the effects of the knockdown of *Bmi-1* on the viability of OVCAR-3 cells, a MTT assay was performed (Fig. 2). Cells treated with *Bmi-1* siRNA showed cellular activity that decreased with time after siRNA treatment compared with untreated cells or cells treated with the negative control siRNA ($P<0.05$). The cells treated with negative control siRNA had cellular activity levels similar to those of the untreated cells ($P>0.05$).

Telomerase activity

To examine telomerase activity in OVCAR-3 cells treated with *Bmi-1* siRNA, telomerase activity was compared to that of human embryo kidney 293 cells, which show a constant positive telomerase activity. The OVCAR-3 cells treated with *Bmi-1* siRNA had significantly lower telomerase activity than untreated cells or cells treated with negative control siRNA ($P\leq0.05$), while untreated OVCAR-3 cells had activity levels

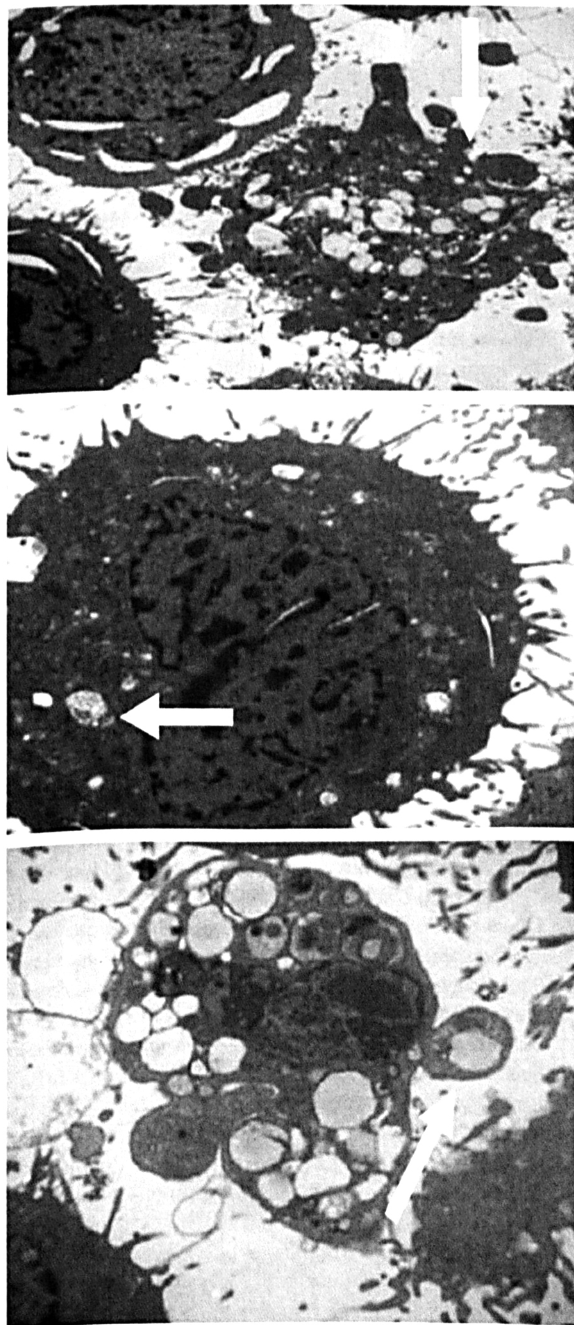

Fig. 4. Apoptotic vacuoles and damaged mitochondria in *Bmi-1* siRNA-treated cells under electronic microscopy. OVCAR-3 cells were treated with *Bmi-1* siRNA. Apoptotic vacuoles and enlarged mitochondria are indicated by white arrows.

similar to that of the human embryo kidney 293 cells (Fig. 3). The untreated cells and cells treated with negative control siRNA showed no significant difference in telomerase activity (P>0.05).

Electron microscopy

Bmi-1 siRNA treatment induced apoptotic vacuoles and damaged mitochondria in the cells. The authors also checked the *Bmi-1* siRNA-induced damage in OVCAR-3 cells using electronic microscopy. This showed the presence of apoptotic vacuoles and damaged mitochondria or other organelles in *Bmi-1* siRNA treated cells (Fig.4). This was not found in the control cells.

Discussion

The silencing of *Bmi-1* with siRNA, as evidenced by RT-PCR and Western blot, led to inhibited cell proliferation and decreased telomerase activity in the ovarian cancer cell line OVCAR-3. Over-expressed *Bmi-1* has been detected in many human cancers.[1,4–16] The expression of *Bmi-1* in ovarian epithelial cancer cases was previously found to be higher than in normal ovarian epithelial tissues, and its expression level relates to the pathological grade and clinical stage of the ovarian cancer.[8] Additionally, ovarian epithelial cancer tissues were demonstrated to have positive telomerase activity, which correlated positively with Bmi-1 protein expression, and was not found in normal tissues. The present study confirms the causative effects of *Bmi-1* on ovarian cell proliferation and telomerase activity, and the results are supported by the inhibited ovarian cancer cell growth observed when *Bmi-1* was knocked down.[20]

The expression of *Bmi-1* appears to be valuable as a prognostic biomarker for ovarian cancer[21] and also for breast,[6] gastric,[22] bladder,[10] nasopharyngeal[12] and pancreatic[14] cancers. Additionally, *Bmi-1* is required for the self-renewal of normal and malignant stem cells.[3] Furthermore, telomerase generally resides in immortalised, malignant and generative cells, and activating telomerase may relate to tumour development.[15] The activity of telomerase correlates with *Bmi-1*,[23,24] and over-expressing *Bmi-1* may induce an increase in telomerase activity in epithelial cells.[4,25] This supports the present work, which suggests that down-regulating *Bmi-1* by RNAi may have therapeutic value in many forms of cancer. Silencing *Bmi-1* may depress telomerase activity and prevent cell immortalisation.

This study is limited because it did not detect any target molecules or the signalling pathway involved in ovarian cancer cell proliferation and telomerase activity. It is unclear whether apoptosis or telomerase mediated the inhibited cellular growth observed with *Bmi-1* siRNA-treated cells. Clearly, the *in vivo* effectiveness of *Bmi-1* siRNA for ovarian cancer requires further study, although it has been shown to inhibit tumourigenicity and enhance radiochemosensitivity in head and neck squamous cell cancer in nude mice.[11]

In conclusion, silencing *Bmi-1* may be a clinical therapy against ovarian cancer and other types of tumour. This study provides independent confirmation of the ability of *Bmi-1* siRNA to inhibit OVCAR-3 cell proliferation. Additionally, it demonstrates that *Bmi-1* siRNA treatment decreases telomerase activity in OVCAR-3 cells, which suggests that silencing *Bmi-1* may be a promising treatment to suppress the development of ovarian cancer. □

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