Correlation of Bmi-1 expression and telomerase activity in human ovarian cancer

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ABSTRACT

This study investigates the correlation between the oncoprotein Bmi-1 and telomerase activity in ovarian cancer. A real-time polymerase chain reaction (PCR) method is used to detect the messenger RNA (mRNA) expression of Bmi-1 protein in 47 ovarian epithelial cancer cases, and immunohistochemistry is used to detect Bmi-1 protein expression in the tissues. A modified telomeric repeat amplification protocol (TRAP) is used to detect telomerase activity. Western blotting is used to detect the expression of telomerase hTERT in the tissues studied. Compared to normal ovarian epithelial tissue, Bmi-1 protein in the 47 ovarian epithelial cancer cases showed higher expression and was related to pathological grade and clinical stage. Significantly higher Bmi-1 levels were found among different clinocopathological types of the cancer ($P<0.05$). Grade G3 cases expressed Bmi-1 at a higher rate (93.10%) than did grade G2 cases (61.11%). Expression in phase II and phase III cases was lower (66.67%) than in phase IV (92.31%). In ovarian epithelial cancer tissues, 87.23% (41/47) cases demonstrated positive telomerase activity, whereas no activity was observed in normal tissues. The majority (90.24%) of specimens with positive telomerase activity showed high Bmi-1 expression levels. Spearman correlation analysis indicated that expression of Bmi-1 protein correlated positively with elevated telomerase activity. Bmi-1 protein is highly expressed in ovarian epithelial cancer tissues, and expression correlates with histological grade and clinical phase. Elevated Bmi-1 expression correlates closely with increased telomerase activity and plays a significant role in the pathogenesis of ovarian cancer.

KEY WORDS: BMI1 protein, human.
Telomerase.
Ovarian neoplasms.

Introduction

Bmi-1 was first identified at The Netherlands Cancer Centre in 1991 and belongs to the polycomb group gene family (PcG).[1] *Bmi-1* over-expression has been found in malignant diseases such as leukaemia,[2] lymphoma,[3] colon cancer, small cell lung cancer and breast cancer.[4–23] More recently, it has been shown that *Bmi-1* is strongly expressed in primary neuroblastomas.[24,25]

Since the identification of telomerase in 1985 by Greider and Blackburn,[5] the biological functions of telomerase have been studied extensively. Human telomerase reverse transcriptase (hTERT) is a catalytic subunit of telomerase that potentially is a useful diagnostic marker for cancer. Telomerase activation correlates directly with cell immortalisation and tumourigenesis, and more than 85% of malignant tumour tissues have been found to show enhanced telomerase expression.[6]

It is suggested that Bmi-1 protein is closely related to telomerase activity,[7] but the presence of Bmi-1 in ovarian cancer tissues has not been reported. In this study, the correlation of Bmi-1 expression, telomerase activity and the effects of Bmi-1 on the genesis and development of ovarian epithelial cancer are investigated.

Materials and methods

Forty-seven patient samples were selected randomly from ovarian epithelial cancer cases admitted to the Department of Gynecology, Affiliated Oncology Hospital of Harbin Medical University, from August 2006 to June 2007. All cases were confirmed as ovarian epithelial cancer by pathological examination. Ten cases of normal ovarian tissue were chosen as controls. Within 30 min of excision, samples of tissue were cultivated and portions of tissue were frozen at –70°C. The remainder was fixed in 10% formalin, processed and embedded in paraffin wax.

Patient age ranged from 30 years to 72 years. Serous papillary adenocarcinoma was diagnosed in 28 cases, and mucoid adenocarcinoma was diagnosed in the remaining 19 cases. Using the International Federation of Gynecology and Obstetrics (FIGO) staging method, nine cases were stage II, 12 cases were stage III and 26 cases were stage IV.

Reagents used were as follows: mouse anti-human Bmi-1 monoclonal antibody (Upstate Company, USA); immunohistochemistry kit and antibody diluent (Beijing Zhongshan, China); Trizol reagent (Invitrogen); Biowest agarose, reverse transcriptase M-MLV, RNase inhibitor, oligo (dT)18, dNTP mixture, SYBR Premix Ex Taq, real-time PCR TP800 (TaKaRa), ECL (Pufei, Shanghai, China).

Primer sequences used were as follows: 5′-AATGTCTTTGGATTTGGGAATCTTAT-3′ (forward); and 5′-TGGTCTAACCAGAGAGACCCAGTA-3′ (reverse).

Primers for *Bmi-1* were those reported by Kim *et al*.[4] and were as follows: 5′-AATCCGTCGAGCAGAGTT-3′ (forward);

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and 5′-CCCTTACCCTTACCCTTACCCTTAA-3′ (reverse). Primers were synthesised by Shanghai Bioengineering, China.

A real-time polymerase chain reaction (PCR) method was used to detect the expression of Bmi-1 protein in tissue samples. Briefly, total RNA was isolated from cell culture media using Trizol reagent and complementary DNA (cDNA) was generated from 20 μL of total RNA using reverse transcriptase M-MLV reagent, following the manufacturer's instructions (Tables 1, 2 and 3). The conditions used were as follows: degeneration at 95°C for 10 sec, annealing at 57°C for 20 sec, prolonged for 40 cycles of 72°C for 20 sec, and a final step at 72°C for 5 min. The product was visualised by agarose gel electrophoresis at 100 V for 30 min.

Immunohistochemical examination was undertaken on serial sections (4 μm). Briefly, sections were dewaxed (x2) in dimethyl benzene for 10 min each, and dehydrated in an ethanol gradient. Endogenous peroxidase was removed using 3% H_2O_2 and the tissues were treated with EDTA (pH 8.0) repair liquid. Sections were exposed to high heat for 5 min and then cooled for 2 min at room temperature (RT). This was followed by 20 min at medium and low heating to fully expose the antigens. After cooling at RT, solution A from the immunohistochemistry kit was added to the sections and incubated for 15 min at RT, followed by overnight incubation with mouse anti-human Bmi-1 antibody at 4°C. Sections were then washed in 0.1% Tween in 1x phosphate-buffered saline (PBST, x3) for 5 min each. Solution B from the kit was added to the slides and incubated for 15 min at RT, followed by washing with PBST (x3) for 5 min each. Solution C was added and slides were incubated for 15 min at RT, followed by washing with PBST (x3). The sections were then developed with DAB for 10 min, counterstained with haematoxylin, fixed with ethanol hydrochloride, and then mounted. Primary antibody was omitted for the negative control.

The modified TRAP silver staining was performed as described previously.[8] Briefly, for protein isolation, 200 μL prechilled lysis buffer (10 mmol/L Tris-HCl [pH 7.5], 1 mmol/L $MgCl_2$, 1 mmol/L EGTA, 0.1 mmol/L PMSF, 5 mmol/L β-mercaptoethanol, 0.5% CHAPS, 10% glycerine) was added to 40–80 mg tissue from each specimen. This was homogenised in an iced bath and placed on ice for 30 min before centrifugation for 20 min at 16,000 rpm at 4°C. The supernatant was transferred to a new tube and the protein concentration was determined. Protein samples were aliquoted and stored at –70°C.

Table 1. Mixed solution.

Total RNA	2 μg
Oligo(dT)18 primer (50 μmol/L)	2 μL
RNase-free distilled H_2O	up to 10 μL

Table 2. Reverse transcription reaction solution.

RNA	10 μL
5 x M-MLV buffer	4 μL
dNTP mixture (every 10 mmol)	1 μL
Ribonuclease Inhibitor (40 U/μL)	1 μL
RTase M-MLV (RNase H^-) (200 U/μL)	1 μL
RNase-free distilled H_2O	up to 20 μL

Table 3. Constituents of amplification system.

Takara SYBR Premix Ex Taq	10 μL
Primer F (10 μmol/L)	0.4 μL
Primer R (10 μmol/L)	0.4 μL
cDNA	2 μL
Total	20 μL

For the TRAP-PCR reaction, a 25 μL total reaction volume was prepared, which included 20 mmol/L Tris-HCl (pH 8.0), 1.5 mmol/L $MgCl_2$, 63 mmol/L KCl, 0.005% Tween-20, 1 mmol/L EGTA, 50 μmol/L dNTP, 0.1 μg forward and reverse primers, 1 unit *Thermus aquaticus* (*Taq*) polymerase and 0.5–1 μL telomerase extract (5 μg total protein). Amplification conditions were as follows: 25°C for 30 min, 94°C for 2 sec, followed by 30 cycles at 94°C for 30 sec, 50°C for 30 sec, 72°C for 90 sec and a step of 72°C for 10 min. The PCR products were separated on a 12% native polyacrylamide gel and stained with silver. Cellular extract of cell line 293, which showed constant positive telomerase activity, was adopted as the positive control, and cell-free lysis buffer was used as the negative control.

For Western blot analysis, small pieces of tissue were lysed in buffer, centrifuged and the supernatant was used to determine total protein content. Equal amounts of sample proteins were subjected to SDS PAGE, transferred to polyvinylidene difluoride (PVDF) membranes, and the blots

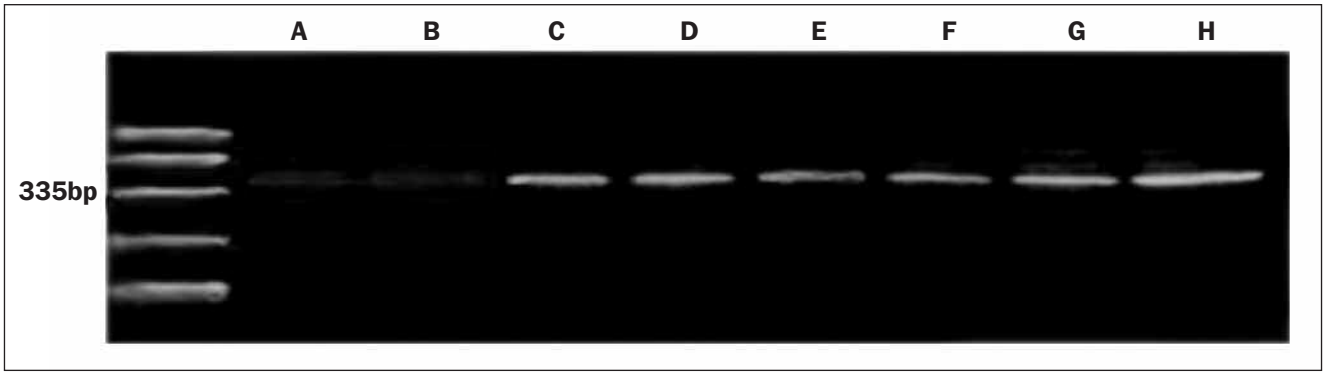

Fig. 1. Expression of Bmi-1 mRNA. Lanes A and B: normal tissues; lanes C–H: ovarian cancer. High expression at 335 bp.

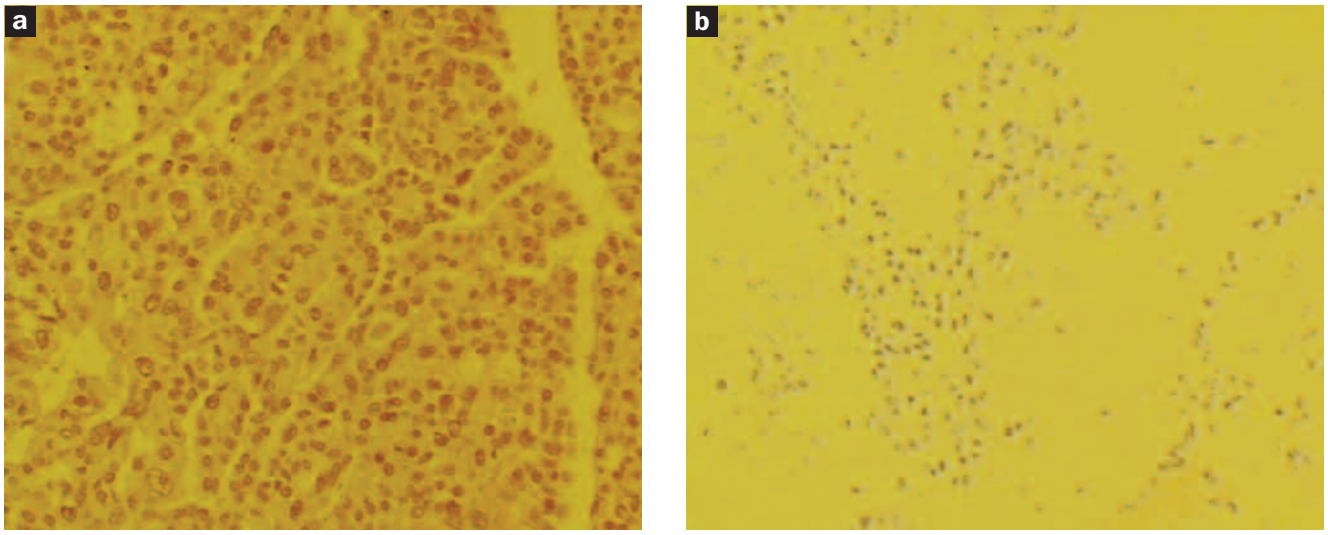

Fig. 2. Expression of Bmi-1 protein in ovarian epithelial cancer. a) Typical histochemical staining showing positive Bmi-1 protein expression as yellow/brown granules in the nuclei (original magnification x400). b) A sample negative for Bmi-1 expression
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were incubated with specific hTERT antibody for 2 h at RT. The blots were washed with buffer and exposed to horseradish peroxidase (HRP)-conjugated IgG. The immunostained proteins were visualised using enhanced chemiluminescence (ECL) reagent.

Positive expression of Bmi-1 was defined as the presence of yellow/brown granules in the nuclei. Occasional yellow/brown granules could also be seen in the cytoplasm. Bmi-1 expression was counted and scored according to the percentage of positive cells as follows: less than 10% positive cells (1 point); 10–50% (2 points); 50–75% (3 points); and >75% (4 points). Staining intensity was scored as follows: negative (1 point); weak staining (2 points); medium staining (3 points); and strong staining (4 points). The scores were combined and interpreted as follows: ≤ 4 points was marked as (–); 4–8 points was marked as (+); 8–12 points was marked as (++); and 12–16 points was marked as (+++). For statistical analysis, (–) and (+) were regarded as negative and weak positive, respectively, while (++) and (+++) were regarded as strong positive. Results were assessed by at least two observers.

Statistical analysis was conducted with SPSS 13.0 (SPSS, Chicago, IL, USA). Data were analysed using the $\chi 2$ test. Correlation analysis was performed for exact probability and enumeration (Spearman correlation analysis). $P<0.05$ was regarded as significant.

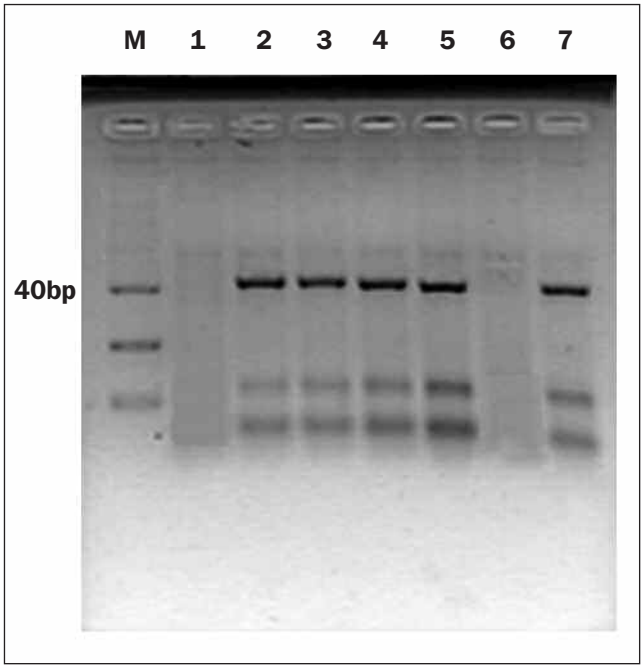

Fig. 3. Telomerase activity in ovarian epithelial cancer. Lane M: positive control; lane 1: negative control; lanes 2–7: tissue samples from six selected cases.

Results

Real-time PCR results for normal tissues and cancer tissues were 0.779±0.112 and 2.607±0.114, respectively ($P<0.05$, Fig. 1). Immunohistochemical examination of the 47 ovarian epithelial cancer specimens revealed that the positive expression rate of Bmi-1 was 80.85% (38/47), mainly concentrated in the nuclei of tumour cells (Fig. 2). The strong positive (+++) rate of Bmi-1 expression was 46.81% (22/47).

Bmi-1 expression of grade G3 was higher than that of grade G2 (Table 4), and the difference was significant ($\chi 2=7.3423$, $P<0.01$). Expression rate for clinical stages II and III was 66.67%, and for stage IV was 92.31% ($\chi 2=4.46$, $P<0.05$). No significant difference in Bmi-1 expression was found between serous and mucous tumour cases ($\chi 2=0.0747$, $P>0.05$).

A representative result for the modified TRAP method is shown in Figure 3. Table 5 summarises telomerase activity data for ovarian epithelial cancer and normal ovarian epithelial tissues. The positive rate of telomerase activity was 87.23% (41/47) for ovarian epithelial cancer samples, while no expression was observed in normal ovarian epithelial tissues. No significant difference was observed between serous and mucous tumour cases ($\chi 2=0.1437$), G2 and G3 grades ($\chi 2=0.0717$) or stage II–III and IV ($\chi 2=0.0787$) specimens ($P>0.05$).

Positive Western blot results were found in 82.98% (39/47)

Table 4. Expression of Bmi-1 protein in epithelial ovarian cancer of various clinopathological types.

	–	+	++	Total	Positive (%)	Strong positive (%)
Serous ovarian cancer	5	10	13	28	82.15	46.43
Mucinous ovarian cancer	4	6	9	19	78.95	47.37
Histological grade						
G2	7	6	5	18	61.11	27.78
G3	2	10	17	29	93.10	58.62
Clinical stage						
II & III	7	8	6	21	66.67	28.57
IV	2	8	16	26	92.31	61.54

Table 5. Telomerase activity in epithelial ovarian cancer of various clinopathological types.

	Total	Telomerase activity		
		Neg	Pos	Positive (%)
Serous ovarian cancer	28	4	24	85.71
Mucinous ovarian cancer	19	2	17	89.47
Histological grade				
G2	18	2	16	88.89
G3	29	4	25	86.21
Clinical stage				
II & III	21	3	18	85.71
IV	26	3	23	88.46
Total cases	47	6	41	87.23

cases (Fig. 4). Correlation of telomerase activity and Bmi-1 protein expression showed significant differences in Bmi-1 protein expression levels between cases with positive and negative telomerase activity ($P<0.01$, Table 6). In tissues with positive telomerase activity, Bmi-1 expression was high (90.24%, 37/41). In telomerase-negative cases, Bmi-1 protein expression was much lower (16.67%, 1/6). Spearman correlation analysis indicated that Bmi-1 expression and telomerase activity were positively correlated ($P<0.05$).

Discussion

Bmi-1 belongs to the polycomb gene family, which includes *RAE28, Bmi-1* and *EZH2*, and controls gene activities during body growth. Bmi-1 is an extensively expressed neucleoprotein and can act as a transcription factor providing specific undisturbed inhibition of targeted gene promoters (e.g., HOX).[9] Bmi-1 expression is associated with the characteristics of normal and tumour stem cells, maintaining the normal size of the stem cell pool.[10,11] Recently published data indicate that an acute small interfering RNA-mediated knockdown of Bmi-1 can result in apoptosis in cancer cells but not in normal cells.[22] Cells with over-expressed Bmi-1 have been implicated as 'tumour stem cells' in carcinomas.[12]

Studies suggest that *Bmi-1* is a proto-oncogene involved in the genesis of various tumours (e.g., B-cell lymphoma, tumours of the nervous system). It has been demonstrated that *Bmi-1* is a target gene for SALL4, and that SALL4 is expressed constitutively in human leukaemia cell lines and primary acute myeloid leukaemia (AML) cells.[23]

Bmi-1 was first proposed as an oncogene in the passage of lymphoma cells in transgenic mice,[13] where researchers found a synergistic effect between *Bmi-1* and *c-myc*, and *Bmi-1* induced cellular transformation and tumor formation. Recently, data have shown that *Bmi-1* is a c-Myc target and its promoter region contains a functional E-box by which *c-myc* and *Mel-18* regulate expression of Bmi-1 during cellular senescence in human cells.[26]

Bmi-1 is reported to induce the immortalisation of breast epithelial cells,[14] and is over-expressed in nasopharyngeal carcinoma.[15] *Bmi-1* alone can induce the immortalisation of nasopharyngeal epithelial cells, and its expression correlates with the infiltration range, survival and prognosis of nasopharyngeal carcinoma.[15] These data provided powerful evidence that *Bmi-1* is an oncogene.

In the present study, *Bmi-1* was up-regulated in serous and mucous ovarian cancers and RT-PCR and

Table 6. Correlation of telomerase activity and Bmi-1 expression.

	Bmi-1 protein	
	Positive	Negative
Telomerase activity positive	37/41 (90.24%)	4/41 (9.76%)
Telomerase activity negative	1/6 (16.67%)	5/6 (83.33%)

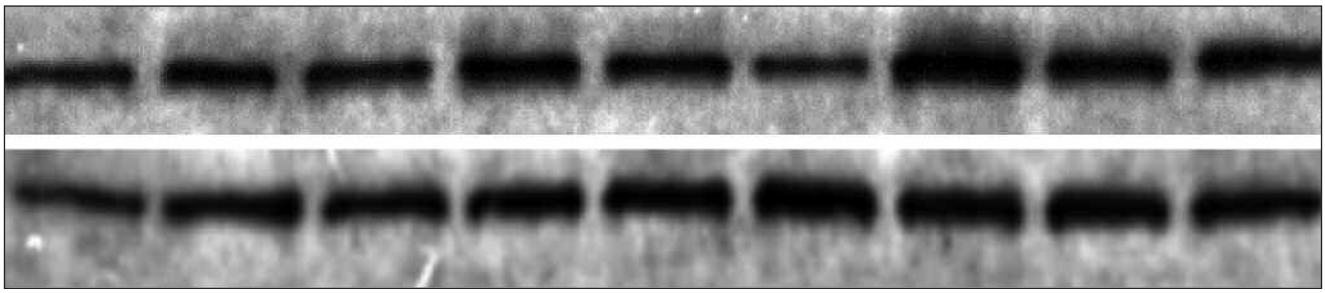

Fig. 4. Western blot analysis of hTERT expression in ovarian cancer. Band shown at 47 kDa.

immunohistochemistry results coincide. Bmi-1 expression in poorly differentiated tumours was notably higher than that in well-differentiated tumours, and correlated with clinical phase. These results suggested that Bmi-1 plays an important role in ovarian cancer progression.

Telomerase is a nuclear ribonuclear protein that elongates the telomere. Once activated, telomerase can overcome shortening of the telomere during cell cycling. Telomerase activation is crucial in the genesis and development of tumours,[6] as has been demonstrated in ovarian cancers.[16,17] Results in the present study indicated close and positive correlation of Bmi-1 expression and telomerase activity. Over expression of Bmi-1 was clearly seen in tissues with positive telomerase activity, and is consistent with previous similar studies.[7,18]

Dimri *et al*.[14] observed over-expression of Bmi-1 protein in immortalised breast epithelial cells and breast cancer cells, and that the oncogene *Bmi-1* could increase cell replication and lead to cell immortalisation. Takeda *et al*.[18] found that *Bmi-1* regulates the human telomerase reverse transcriptase (hTERT) promoter via the *c-myc* pathway. Furthermore, peptide deletion analysis of the Bmi-1 protein indicates that the ring finger and helix-turn-helix structures are necessary to induce telomerase activation and immortalisation of epithelial cells.

Over-expression of *Bmi-1* in breast epithelial cells is thought to activate hTERT transcription and further induce telomerase activation.[19] In another study, over-expression of *Bmi-1* was found to prolong multiplication and block apoptosis of cells through inhibition of $p16^{INK4a}$ expression.[20] Based on these results, the present findings suggest correlation with Bmi-1 oncoprotein activation, participation in cellular multiplicative division and down-regulation of $p16^{INK4a}$, which prolongs the life of ovarian epithelial cancer cells by promoting proliferation and suppressing apoptosis. Meanwhile, *Bmi-1* could activate hTERT transcription and induce telomerase activation, thereby promoting immortalization of the relevant cells.

Data suggest a positive correlation between Bmi-1 expression and telomerase activity in ovarian epithelial cancer tissues. Bmi-1 is presumed to play important roles in ovarian epithelial cancer progression and regulation of telomerase activation. Thus, blocking Bmi-1 expression could decrease telomerase activity, which would provide a new direction for the genetic treatment of ovarian cancer. However, further studies of the regulation of telomerase activation by Bmi-1 are required. □

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