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Original Article

Over-expression of BMI1 is associated with favorable prognosis in cervical cancer

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Abstract: Objective: B cell-specific Moloney murine leukemia virus integration site 1 (BMI1), is a Plycomb group (PCG) protein, that is involved in the epithelial-mesenchymal transition (EMT) and induces stem cell properties, suggestive poor prognosis. In this study, we evaluated the prognostic value of BMI1 in cervical cancer. Methods: The study materials were comprised of cervical intraepithelial neoplasia (CIN, n=225), cervical cancer (n=150) and matched nonadjacent normal tissues (n=326). In order to identify BMI1 expression in the tissues, immunohistochemistry (IHC) was performed. IHC scoring was performed using digital image analysis, and the associations of BMI1 with prognosis, radiation sensitivity and human papillomavirus level were examined. Results: BMI1 compared with normal cervix and CIN lesion was highly expressed in cervical cancer. High expression of BMI1 presented better disease-free survival and overall survival than low expression according to a Kaplan-Meier survival analysis (P=0.017 and 0.035, respectively), and produced a significantly low hazard ratio for death according to a multivariate analysis (P=0.03). In CIN lesion, BMI1 was correlated with cancer stem cell (CSC) markers such as OCT4 and SOX2 (P=0.006 and 0.031, respectively), whereas in cervical cancer, no association was observed. Additionally, BMI1 expression was observed in radiation-sensitive cervical cancer, suggesting its positive prognostic indication. Conclusions: BMI1 expression is associated with favorable survival in cervical cancer, and as such, might aid the prognosis of cervical carcinoma.

Keywords: BMI, cervical cancer, prognosis

Introduction

Cervical cancer is the fourth most common malignancy in women worldwide; indeed, it remains a leading cause of women's death [1]. Although cervical cancer can be detected early thanks to advanced screening systems and the fact that is symptomatic at early stages, advanced cases requiring multimodal treatment including chemotherapy, radiation and other modalities continue to be diagnosed. Chemo-radiation therapy has produced favorable responses in patients with advanced cervical cancer. However, some cancer cells acquire resistance to chemo-radiation; difficult to fully eradicate, they are often the eventual cause of death. Cancer cells that are resistant to chemotherapy and radiotherapy have properties of cancer stem cells (CSCs) that are implicated in treatment failure [2, 3].

B cell-specific Moloney murine leukemia virus integration site 1 (BMI1) is one of the Polycomb group (PCG) proteins, that are involved in chromatic modification, suggesting cancer development and maintenance of embryonic and adult stem cells [4]. BMI1 plays an important role as a transcriptional regulator through chromatin modification, and is involved in cell-cycle regulation, hematopoiesis and senescence [5, 6]. Through chromatin and histone modification, BMI1 regulates cell cycle and self-renewal, specifically by suppressing the INK4a locus that inactivates tumor suppressors p16 and $p14^{ARF}$ [7, 8].

BMI1, unfortunately, is dysregulated in cancer cells; this is responsible for invasiveness and poor prognosis in various cancers [9]. BMI1 is involved in the epithelial-mesenchymal transition (EMT), which enhances metastasis and

Table 1. Patients' clinicopathologic characteristics

	Frequency	%
Age	43.9*	
Diagnostic category		
Normal	323	46.3
CIN	225	32.2
Cervical cancer	150	21.5
FIGO stage		
<IIA	108	72
>IIB	42	28
Tumor differentiation		
Well to Moderate	108	73.5
Poor	39	26.5
Cell type		
Squamous cell carcinoma	119	79.3
Adenocarcinoma	15	10
Others	16	10.7
Tumor size		
≤4 cm	100	66.7
>4 cm	50	33.3
Lymphovascular invasion†		
No	68	51.1
Yes	65	48.9
Lymph node metastasis‡		
No	97	71.9
Yes	38	28.1

CIN, cervical intraepithelial neoplasia; FIGO, International Federation of Gynecology and Obstetrics; *mean value; †calculated only 133 cases with available information on examined lymphovascular invasion, ‡calculated only 135 cases with available information on examined lymph node.

induces cells with stem-like properties [9, 10]. BMI1 cooperates with Snail or Twist1, key regulators of EMT, and promotes EMT through suppression of PTEN/E-cadherin expression or repression of p16INK4a [9, 11, 12]. Cancer cells undergoing EMT also acquire cancer stem cell (CSC) properties, suggesting a crucial association [10]. BMI1 enhances tumorigenesis and stem cell properties and shows a correlation with stem cell markers in various cancer cells [13, 14]. In cisplatin-resistant oral squamous cell carcinoma, CSC properties are expanded, and BMI1 is highly expressed with stem cell markers including OCT4 and Nanog [15]. Also, BMI1 is a key mediator of SOX2 function, the connection between BMI1 and SOX2 being essential to self-renewal [16].

BMI1 expression is increased in various cancers including lung, colorectal, breast and oral, and its high expression is associated with poor prognosis [14, 17-19]. In light of such accumulating evidence, it is expected that BMI1 plays a key role in tumorigenesis and cancer prognosis. However, its prognostic significance has been the subject of controversy, as improved survival outcomes have been reported for glioblastoma, breast and colorectal cancer patients showing high BMI1 expression [20-22]. Furthermore, prognosis of BMI1 in cervical cancer is not yet firmly established. In this study, we evaluated the protein expression of BMI1 by digital image analysis, on which basis we investigated the prognostic significance of BMI1 in cervical cancer.

Materials and methods

Patient' selection

A total of 375 patients with cervical cancer and cervical intraepithelial neoplasia (CIN) along with 323 matched normal patients from Gangnam Severance Hospital, Yonsei University College of Medicine in Seoul, Korea and the Korea Gynecologic Cancer Bank were assessed as part of the Bio & Medical Technology Development Program of the Ministry of Education, Science and Technology, Korea, between 1996 and 2010. Medical records were reviewed for patient data including age, cancer stage, tumor differentiation, cell type, tumor size, lymphovascular invasion (LVI) and lymph node (LN) metastasis. The cervical cancers were histologically classified and graded according to the WHO classification and staged according to the International Federation of Gynecology and Obstetrics (FIGO) stage. Radical hysterectomy with pelvic and aortic LN dissection in patients with operable indications, and concurrent chemoradiation therapy, were added to the risk factors including LN metastasis, parametrial invasion and positive resection margin. The patients with inoperable conditions underwent radiation or chemoradiation therapy. Those who recurred within 1 year of radiation or chemoradiation therapy were regarded as radiation sensitive. The current study was approved by the Institutional Review Board of Gangnam Severance Hospital.

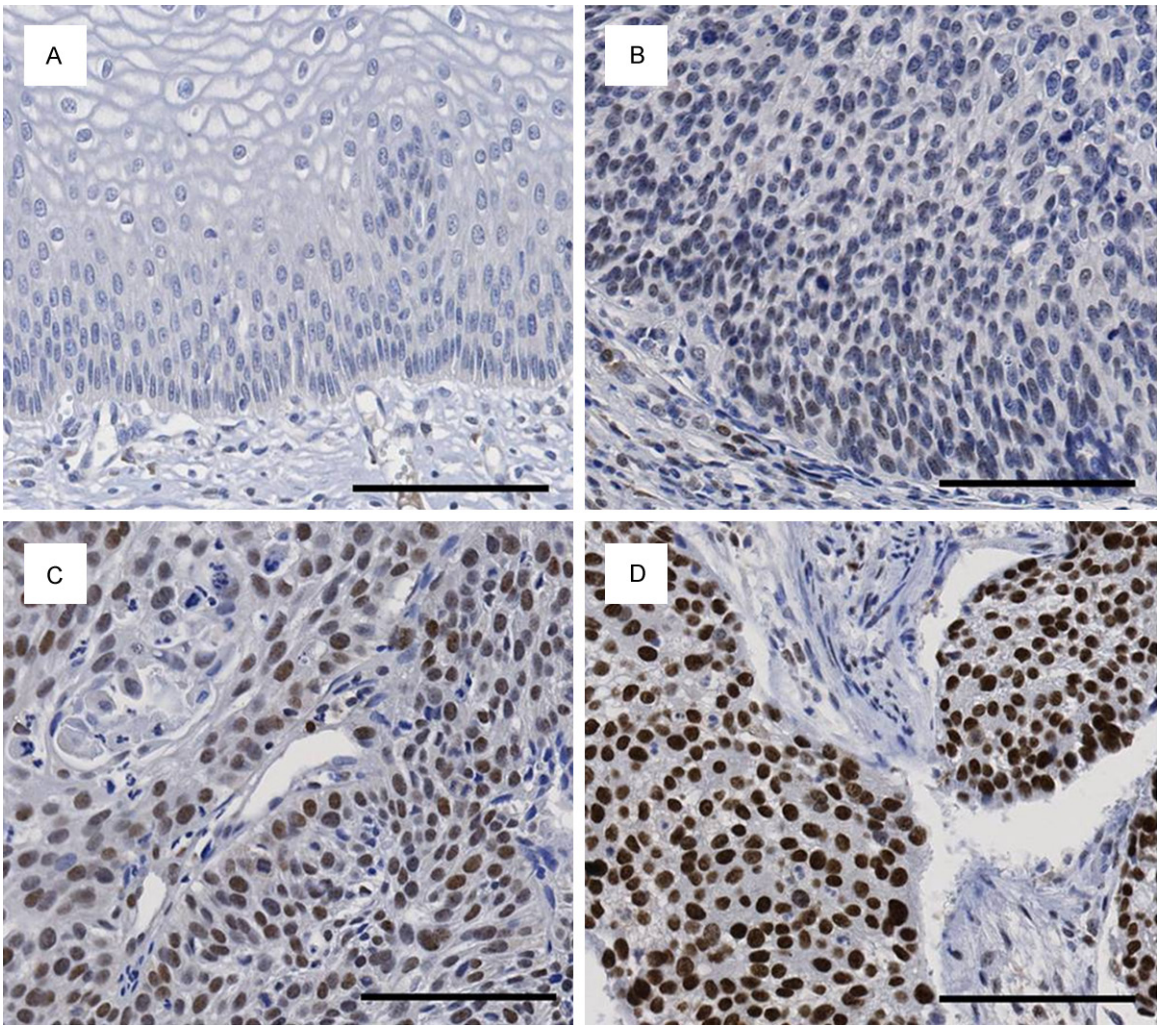

Figure 1. Representative IHC expression of BMI1. BMI1 expression showing (A) no nuclear staining in normal cervix, (B) weak nuclear staining in CINII, (C) moderate nuclear staining in squamous cell carcinoma, and (D) strong nuclear staining in squamous cell carcinoma. Scale bar: 100 μm.

Tissue microarray construction and immunohistochemistry

Tissue microarrays (TMAs) were constructed from 375 formalin-fixed paraffin-embedded tissue specimens. Meanwhile, 323 nonadjacent normal tissues were prepared as well. After the slides were reviewed by a pathologist, areas containing each category were indicated on H&E slides. 1 mm punches were then taken from the corresponding regions of the paraffin blocks and transplanted into a recipient paraffin block using a tissue arrayer (Pathology Devices, Westminster, MD).

For IHC staining, all TMA sections were cut to 5 micron thickness followed by deparaffinization through xylene and dehydration with graded ethanols. Antigen recovery was performed in heat-activated antigen retrieval pH6 (Dako, Carpinteria, CA) for BMI1, after which specimens were incubated with 3% H_2O_2 for 10 min. Non-specific binding was blocked with protein block (Dako) for 20 min at room temperature. The sections were then incubated with anti-BMI1 antibody (Sigma-Aldrich, St. Louis, MO) at 1:100 for 1 hour. Subsequently, sections were incubated with DAKO Env+ secondary antibody for 30 min, visualized with 3,3-diaminobenzadine for 10 min for chromogenic development, and washed and counterstained with hematoxylin. Negative controls were concurrently performed. The positive controls included normal cervix, esophageal and gastric epithelium for the BMI1 antibody.

Table 2. Association of BMI1 expression with clinicopathologic characteristics

	N	Mean Histoscore (95% CI)	*P*
Diagnostic category			<0.001
Normal	323	31.2 (27.3-35.1)	
CIN	225	35.6 (29.3-42.9)	
Cervical cancer	150	62.5 (50.6-74.5)	
FIGO stage			0.256
I	98	69.2 (53.5-85.1)	
II	46	47.5 (29.1-65.8)	
IV	6	69.1 (5.4-143.7)	0.770
Tumor differentiation			
Well + Moderate	88	64.2 (48.0-80.3)	
Poor	59	60.5 (41.6-79.3)	
Cell Type			0.456
Squamous cell carcinoma	119	60.2 (46.9-73.6)	
Others	31	71.4 (43.0-99.9)	
Tumor size			0.391
<4 cm	100	66.2 (50.8-81.7)	
≥4 cm	50	55.2 (36.2-74.1)	
Lymphovascular invasion			0.429
Negative	68	59.0 (40.8-77.2)	
Positive	65	69.5 (50.3-88.7)	
Lymph node metastasis			0.347
Negative	97	67.3 (51.6-82.9)	
Positive	38	53.6 (30.5-76.7)	

CI, confidence interval; CIN, cervical intraepithelial neoplasia; FIGO, International Federation of Gynecology and Obstetrics.

Digital image analysis

Immunohistochemically stained whole sections were digitized at 20 × magnification and 0.50 μm/pixel spatial resolution utilizing an Aperio Scanscope CS (Aperio, Vista, CA,). The images were reviewed utilizing an online software application, Digital Image Hub (SlidePath, Dublin, Ireland), that enables users to annotate normal and tumor regions. Once the regions were annotated, they were sent for automated image analysis utilizing Tissue IA (SlidePath's Tissue IA system, version 3.0, Dublin, Ireland), which includes an algorithm developed for quantification of BMI1. Briefly, BMI1 nuclei were stained, the intensity of which was categorized as 0 (no staining), 1+ (weak), 2+ (moderate) or 3+ (strong). BMI1 were interpreted according to weighted histoscores (i.e., multiplied by the percentage of nuclear intensity) ranging from 0 to 300: Histoscore = (0 × percentage not stained) + (1 × percentage weakly stained) + (2 × percentage moderately stained) + (3 × percentage strongly stained) [23]. To investigate the association of BMI1 with CSC markers, OCT4 and SOX2 protein expressions, stained previously at this institution (data unpublished), were used.

Statistical analysis

The IHC scores were compared by one-way ANOVA and independent t-test. The histoscore cut-off for high expression of tumor markers was determined by receiver operating characteristic (ROC) curve analysis. The sensitivity and (1-specificity) for discriminating death or survival was determined for each IHC score and plotted, thus generating the ROC curve. The cut-off value was determined to be the point on the ROC curve where the sum of sensitivity and specificity was maximized. Kaplan-Meier survival analysis was performed to determine the associations of BMI1 expression with disease-free and overall survival, and the survival curves were compared between the groups using log-rank tests. Multivariate analyses of the hazard ratio for death were performed using Cox proportional hazards regression. Chi square testing and Spearman's rank correlation were used to evaluate the associations of BMI1 with CSC markers and radiation sensitivity. Statistical analyses were performed using SPSS version 18.0 (SPSS Inc., Chicago, IL). A value of $P<0.05$ was considered statistically significant.

Results

Clinicopathologic characteristics of patients

The patients' clinicopathologic characteristics are summarized in **Table 1**. There were 225 CIN and 150 cervical cancers. Among the 150 patients with cervical cancer, the number with stage I and IIA was 108, and the number with IIB and IV was 42. The patients' mean age was 43.9 years (range: 21-83 years). The tumor sizes ranged from 0.3 to 12 cm (mean: 3.0 cm). The cervical cancer tumors included 119 squamous cell carcinoma (SCC), 15 adenocarcinoma, 8 adenosquamous and 8 other cell types (2 small cell carcinoma, 2 neuroendocrine, 4 mixed cell types). In a survival analysis of 150 patients, the mean follow-up time for survivors was 59.7 months (range: 1-187), during which 19 patients (12.6%) died.

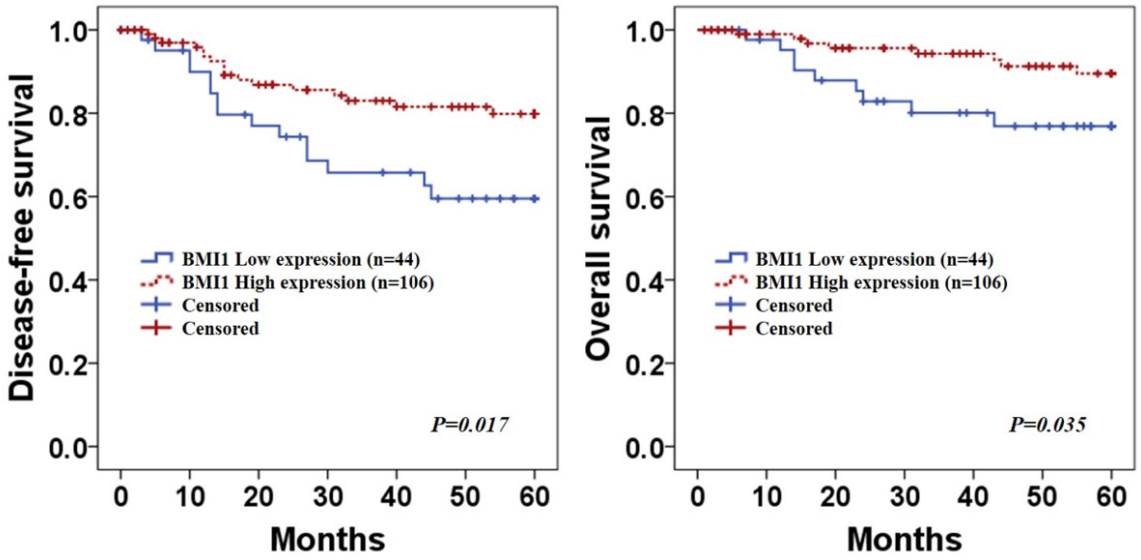

Figure 2. Five-year disease-free and overall survivals analyzed by Kaplan-Meier plot.

Table 3. Multivariate survival analysis of disease-free and overall survival of 150 patients with cervical cancer

	Disease-free survival		Overall survival	
	Hazard ratio [95% CI]	*p value*	Hazard ratio [95% CI]	*p value*
FIGO stage (≥IIB)	5.86 [2.70-12.70]	*<0.001*	3.01 [1.13-8.01]	*0.027*
Tumor size (>4 cm)	0.68 [0.34-1.50]	*0.346*	1.30 [0.28-3.48]	*0.601*
Tumor differentiation (poor)	0.90 [0.44-1.84]	*0.784*	1.26 [0.50-3.21]	*0.616*
BMI1 (high expression)	0.43 [0.21-0.88]	*0.021*	0.34 [0.13-0.90]	*0.030*

Expression of BMI1 protein

Expression of BMI1 in cervical neoplasia was investigated by IHC; the cancer specimens and IHC histoscores were analyzed with digital image software. The representative IHC expression of BMI1 is presented in **Figure 1**. As indicated, BMI1 expression was clearly evident in the nucleus.

Of the 375 patient's specimens, 106 of 150 cancers (70.6%) showed high expression of BMI1, while 167 of 225 CIN (74.2%) showed high expression (cut-off value: 7). The associations of BMI1 expression with the cervical cancer patients' clinicopathologic characteristics are summarized in **Table 2**. As indicated, BMI1 expression in cervical cancer was significantly higher than in normal cervix or CIN lesion (**Table 2**). However, there was no association between BMI1 expression and cancer stage, tumor differentiation, tumor cell type, tumor size, lymphovascular space invasion, or LN metastasis (**Table 2**).

Survival outcome of BMI1 expression

Five-year disease-free and overall survivals were analyzed by Kaplan-Meier plot as shown in **Figure 2**. In the disease-free survival analysis of BMI1, the number of recurrences was 17 of 106 in high-expression patients, while 15 recurrences of 44 low-expression patients were shown. High expression of BMI1 showed a better disease-free survival rate than that of the low-expression group (P=0.017) (**Figure 2A**). In the overall survival analysis for BMI1 expression, 8 deaths in 106 high expressions occurred, while 9 deaths were observed in 44 low expressions. High expression of BMI1 showed a better overall survival rate than that of the low expression group, and significantly (P= 0.035) (**Figure 2B**).

Multivariate survival analysis

Cox proportional multivariate analysis was performed to investigate the association of BMI1 with survival outcome (**Table 3**). The FIGO stage

Table 4. Association of BMI1 with CSC markers and radiation resistance in cervical cancer or CIN

	BMI1 expression in CIN				BMI1 expression in cervical cancer			
	Low (%)	High (%)	No	*p* value	Low (%)	High (%)	No	*p* value
SOX2-low expression	13 (44.8%)	16 (55.2%)	29	0.031	9 (26.5%)	25 (73.5%)	34	0.463
SOX2-high expression	29 (24.8%)	88 (75.2%)	117		30 (29.4%)	72 (70.6%)	102	
OCT4-low expression	24 (41.4%)	34 (58.6%)	58	0.006	13 (25.0%)	39 (75.0%)	52	0.345
OCT4-high expression	56 (27.7%)	146 (72.3%)	202		25 (27.9%)	59 (72.1%)	84	
Radiation sensitive		NA			12 (30.0%)	28 (70.0%)	40	0.049
Radiation resistant					5 (71.4%)	2 (28.6%)	7	

Table 5. Association between BMI1 and HPV titer in cervical cancer

			HPV titer
Spearman's rho	BMI1 Histoscore	*r*	-0.381
		p	0.045
		N	28

and BMI1 showed statistical significances in disease-fee and overall survival. High expression of BMI1 presented a 0.43 hazard ratio for disease-free survival and a 0.34 hazard ratio for overall survival relative to low expression of BMI1 with statistical significance (P=0.021 and 0.03, respectively).

Associations with cancer stem cell markers and radiation sensitivity

To determine the association of BMI1 with stem cell markers SOX2 and OCT4 in malignant and premalignant lesions, a Chi square test was used (**Table 4**). In the CIN lesions, BMI1 presented significant correlations with SOX2 and OCT4 (P=0.031 and 0.006, respectively), whereas in the cervical cancer cases, there were no such associations (**Table 3**). Of the 47 patients who received radiation therapy, those that were radiation sensitive numbered 40. Among these radiation-sensitive patients, the rate of high expression of BMI1 was 70% (28/40), whereas among the radiation-resistant patients it was 28.6% (2/7). The correlation of high BMI1 expression with radiation sensitivity was most significant in cervical cancer (P=0.049).

Additionally, the association between BMI1 histoscore and human papillomavirus (HPV) titer was investigated, results for which are summarized in **Table 5**. In invasive cervical cancer, BMI1 histoscore presented a significantly negative correlation with HPV level by Spearman's rank correlation (P=0.045).

Discussion

This study evaluated the prognostic value of BMI1 in cervical cancer. BMI1 was highly expressed in cervical cancer compared with normal cervix and CIN lesion. In a Kaplan-Meier survival analysis, high expression of BMI1 was associated with favorable disease-free survival and overall survival; according to a multivariate analysis meanwhile, it produced a significantly low hazard ratio for death. BMI1 also was closely associated with CSC markers OCT4 and SOX2 in CIN lesion, whereas there was no such association in cervical cancer. Additionally, BMI1 expression was observed in radiation-sensitive cervical cancer, suggesting that BMI1 presents positive prognostic outcomes.

BMI1, regarded as an oncogene, has been suggested to play a role in cancer stem cells. BMI1 is involved in EMT and induces stem cell properties by cooperating with Snail or Twist1. Overexpression of BMI1 inactivates tumor suppressor genes p16 and $p19^{ARF}$ by suppressing the INK4a locus, eventually regulating apoptosis and senescence [7, 8]. BMI1 expression is observed in various cancers including lung, colorectal, breast and oral, and high expression is expected to be associated with poor prognosis [14, 17-19]. Our present results, contrastingly, presented favorable survival outcomes; in fact, high expression of BMI1 was observed in patients with radio-sensitive cervical cancer. Similarly to our findings moreover, associations of improved survival with high expression of BMI1 have been reported for glioblastoma, breast, and colorectal cancers [20-22]. BMI1 has been posited to have multiple functionalities, among which are oncogenic and tumor-suppressive roles [13, 24]. BMI1 has been reported to dysregulate tumor suppressor genes Rb and p53 by suppressing p16INKa and p19ARF through inactivation of INK4a [5, 6].

Although BMI1 prevents TP53 activation by suppressing INK4a/ARF, TP53 activation is not selectively expressed by BMI1 over-expression; and in any case, other oncogenic activations express INK4a/ARF as part of the tumor-suppressive response. Notably, TP53 activation by DNA damage occurs via ATM/ATR signaling rather than via ARF, which implies that treatment of tumors with BMI1 expression by DNA damage might activate the TP53 pathway to induce apoptosis [25, 26]. Pietersen et al. reported that BMI1 over-expression was associated with favorable prognosis in patients undergoing chemotherapy or hormone therapy for breast cancer [24]. Our result also indicated that BMI1 over-expression is associated with radio-sensitivity and improved survival. Both our data and Pietersen et al's suggest that BMI1 over-expression can result in favorable prognosis in cases of DNA-damaged tumor after chemotherapy or radiation therapy. Furthermore, high expression of BMI1 is closely correlated with expression of trimethylation of lysine 27 on histone H3 (H3K27me3), which involves in epigenetic regulator, and that both H3K27me3 and PCG proteins including BMI1 might prevent aberrant expression of oncogene [20, 27].

HPV is a well known causative agent of cervical cancer, and its infection has been reported to be involved in down-regulation of BMI1 [28]. HPV16 E6 and E7 oncogenes result in down-regulation of the PRC1 protein BMI1, but also induce over-expression of the PRC2 protein EZH2, suggesting progression of HPV 16-associated cancers [28]. Furthermore, BMI1 protein has been negatively correlated with high-risk HPV-positive DNA and mRNA in penile carcinoma [29]. In our results, HPV level also presented a significantly inverse correlation with BMI1 protein expression in cervical cancer. Interestingly, whereas BMI1 is known to suppress p16INK4a expression, a known surrogate marker of CIN, HPV infection induces p16INK4a expression, which fact supports the mostly expressed and known to be a surrogate marker of CIN, supporting BMI1 function's restriction by HPV infection in cervical cancer.

Reports of the association of prognosis with BMI1 expression in cervical cancer have been sparse. In a previous analysis by IHC, high expression of BMI1 was correlated with poor overall survival, contrary to our present result [30]. Although the case of this disparity is not yet clear, interpretation of the results of IHC with different antibodies and cohort tissue samples might produce conflict results. In this study, we evaluated BMI1 protein expression by automated digital image analysis, which offers the advantages of objectivity, reproducibility and quantitative assessment of IHC stains [31, 32]. Although our interpretation method is relatively objective, survival outcome as a correlate with BMI1 expression remains controversial; further investigation of BMI1-related prognosis is required.

BMI1 is well known to be required for the self-renewal of CSCs as a key mediator of SOX2 or cooperator with OCT4 [16, 33, 34]. In our study, the correlation of BMI1 with stem cell markers such as OCT4 and SOX2 was investigated. In CIN lesion, BMI1 was significantly correlated with OCT4 and SOX2, but in invasive cervical cancer, no such association was observed. Kang et al. reported BMI1 expression in the oral mucosal tissue of precancerous and cancerous tissue [12]. Indeed, BMI1 expression occurs, through P16INK4a-independent pathways, at a very early stage of carcinogenesis. For this reason, BMI1 maintains the association with CSCs in premalignant lesion but, in malignant lesion, might lose the association with CSC and its phenotype during tumor progression.

Our survival-prognosis results represent a relatively large number of clinical tissue samples; however, the number of radiation therapy patients was limited. The association between BMI1 expression and radiation sensitivity requires further investigation.

In conclusion, high expression of BMI1 was associated with improved disease-free survival and overall survival in a multivariate analysis. BMI1 was positively correlated with CSC markers OCT4 and SOX2 in CIN lesion, whereas in cervical cancer, no association of this kind was observed. Notably, BMI1 expression was observed in radiation-sensitive cervical cancer, suggesting its positive prognostic indication.

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Disclosure of conflict of interest

None.

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