

Expression and survival significance of B-cell-specific Moloney murine leukemia virus integration site 1 and matrix metalloproteinase-9 in non-small-cell lung cancer

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Abstract. One of the main challenges in lung cancer research is identifying patients at high risk of progression and metastasis following surgical resection. In the present study, the prognostic significance of B-cell-specific Moloney murine leukemia virus integration site 1 (BMI1) and matrix metalloproteinase-9 (MMP9) in non-small-cell lung cancer (NSCLC) was evaluated. BMI1 and MMP9 expression in tumors from 132 surgical NSCLC patients [squamous cell carcinoma (SCC), n=79; and adenocarcinoma (AD), n=53] was evaluated by immunohistochemistry. The clinical significance was determined using multivariate Cox regression analysis, Kaplan-Meier curves and the log-rank test. High BMI1 expression was more frequent in SCC compared with that in AD (P=0.015). Comparisons between the expression of BMI1 and that of other known biological markers revealed that the expression of BMI1 was correlated with that of MMP9 ($\chi^2=4.241$, P=0.039) in SCC. Although an association was not identified between high BMI1 expression and overall survival (OS) in NSCLC or AD, high BMI1 expression was an unfavorable predictor of survival in SCC according to the survival curves (P=0.038). In addition, combined high BMI1 and MMP9 expression levels were significantly correlated with SCC nodal/distant metastasis ($\chi^2=6.392$, P=0.014). Multivariate Cox proportional model analysis demonstrated that this combined marker was an independent prognostic indicator of OS in SCC (P=0.025; hazard ratio = 12.963; 95% confidence interval: 1.142-7.637). Therefore, this study demonstrated that combined BMI1 and MMP9 expression may be used as a marker for the progression and metastasis of

SCC. These results may aid in the elucidation of the potential mechanism underlying the involvement of BMI1 and MMP9 in tissue-specific SCC progression.

Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide, with ~1.4 million deaths recorded annually (1). Approximately 80% of all lung cancers are classified as non-small-cell lung cancer (NSCLC), which may be divided phenotypically into two principal subtypes, namely squamous cell carcinoma (SCC) and adenocarcinoma (AD). SCC and AD are often treated similarly; however, they differ not only pathologically, but also in terms of gene expression levels, clinical manifestation and response to targeted therapeutic agents (2-4). Early detection is key to prolonging patient survival; thus, it is crucial to identify molecular markers that predict prognosis and design novel treatment strategies for SCC and AD (5).

The B-cell-specific Moloney murine leukemia virus integration site 1 (BMI1) gene, a member of the polycomb-group (PcG) proteins, was first isolated as an oncogene that acts synergistically with c-Myc in the oncogenesis of murine lymphomas (6,7). BMI1 functions as a transcriptional repressor of BMI1 and c-Myc target genes via an epigenetic mechanism (8). In addition, deregulation of BMI1 gene expression leads to cell proliferation and tumor progression (8,9). Aberrant BMI1 expression has been associated with a number of solid and hematological malignancies (10-18), including NSCLC (19-28). Although the clinicopathological and prognostic significance of high BMI1 expression in NSCLC has been investigated, the results have been inconsistent. For example, certain studies suggested that BMI1 overexpression is associated with poor prognosis in SCC (26); however, Vrzalikova *et al* (22) found no correlation between BMI1 expression and overall survival (OS) in their cohort, which had a preponderance of SCCs.

Matrix metalloproteinase-9 (MMP9), a member of the MMP class of gelatinases, plays a major role in the invasiveness and metastasis of NSCLC, mainly through specific induction by vascular endothelial growth factor receptor-1 (VEGFR-1) (29,30). Jiang *et al* (31) reported that BMI1 activated nuclear factor- κ B (NF- κ B) and subsequently upregulated MMP9 expression, leading to increased migration and invasion of glioma cells;

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Li *et al* (32) demonstrated that BMI1 contributed to the invasion and metastasis of hepatocellular carcinoma by increasing MMP9 and VEGF expression via the phosphoinositide-3-kinase/protein kinase B (PI3K/Akt) pathway. However, the number of studies on the interaction between BMI1 and MMP9 in NSCLC progression and metastasis is limited.

In this study, a survival analysis of BMI1, MMP9 and their combined expression as a marker in lung SCC and AD samples was performed. The aim was to determine whether the combined high BMI1 and MMP9 expression levels predict poor survival in SCC. These results may help elucidate the mechanism underlying tumor progression and metastasis in patients with NSCLC and ultimately identify novel diagnostic and/or prognostic markers.

Patients and methods

Patients and histopathology. The study samples consisted of paraffin-embedded lung tissue samples from 135 patients diagnosed with NSCLC who had undergone surgery between January, 2008 and January, 2009 at the Second Affiliated Hospital of Harbin Medical University (Harbin, China). According to the World Health Organization guidelines (33), the cases were classified as SCC or AD. In total, 132 cases were analyzed (AD, n=79; and SCC, n=53). Of the 132 cases, 10 were well-differentiated, 57 were moderately and 65 were poorly differentiated. The pathological tumor stage distribution was I-IV according to the American Joint Committee on Cancer (34). All the patients were followed up until October, 2013 or until death. The median follow-up duration for survivors was 43.15 months (range, 1.30-62.40 months). The patient population comprised 89 men and 43 women (male:female ratio, 2.07:1) and the median age was 61 years (range, 34-80 years). All the cases representing a spectrum of NSCLC were retrieved from the records of the Second Affiliated Hospital of Harbin Medical University. The Ethics Committee of the hospital granted permission for the study.

Immunohistochemical (IHC) evaluation. The expression of BMI1 and MMP9 in the 132 patients with NSCLC was investigated. Paraffin-embedded sections were processed as previously described (25,27). Briefly, the sections were deparaffinized in xylene and rehydrated in a graded series of ethanol. The sections were subsequently submerged in ethylene diamine tetraacetic acid (pH 8.0) and autoclaved at 121°C for 5 min to retrieve antigenicity. Endogenous peroxidase was quenched with 3% H₂O₂ for 15 min. Following washing with PBS, the sections were incubated with anti-BMI1 (dilution, 1:80; LS-C98480; LifeSpan Biosciences, Seattle, WA, USA) and anti-MMP9 (dilution, 1:800; ab38898; Abcam, MA, USA) antibodies overnight at 4°C. The sections were incubated with peroxidase-conjugated streptavidin for 30 min and the reaction products were visualized with the chromogen diaminobenzidine and counterstained with commercial hematoxylin. The percentage of positive cells was determined by counting 500 cells in five random fields per section. Nuclear and cytoplasmic immunostaining was evaluated using semi-quantitative assessment, in which the percentage of positive cells was calculated (25). The percentage of positive cells was determined by counting 500 cells in five random areas

per section. IHC staining of the cells was scored according to the following criteria: -, no staining; +, 1-10%; ++, 10-50%; +++, 50-75%; and +++, 75-100% cells stained. The IHC scores for BMI1 and MMP9 expression were classified as low (- and +) and high (++, +++ and ++++).

Statistical analysis. The association between factors was evaluated using Pearson's χ^2 test. Cumulative OS curves were plotted using the Kaplan-Meier method and log-rank statistics were used to determine differences between groups. Covariates that remained significant through the univariate analysis were selected for the multivariate analysis. Cox regression was used for the multivariate analysis, with a backward stepwise elimination model. P<0.05 was considered to indicate statistical significance. Two-sided tests were used throughout. Statistical calculations were performed using SPSS 19.0 software (IBM SPSS, Armonk, NY, USA).

Results

BMI1 is overexpressed in NSCLC tissues. On IHC analysis, the majority of cells in NSCLC tissues, including SCC and AD cells, were strongly stained, indicating high expression of BMI1. BMI1 was highly expressed in 95/132 NSCLC cases (72.0%) and 63/79 SCC cases (79.7%) (Fig. 1). The expression of BMI1 in SCC was higher compared with that in AD (79.7 vs. 60.4%, respectively; P=0.015). BMI1 was highly expressed in 32/53 (60.4%) AD cases. Similar to BMI1, MMP9 expression was detected in NSCLC tissues and its expression level was higher in SCC compared with that in AD (P=0.012, Fig. 1).

Correlations of BMI1, MMP9 and combined marker expression with variables in NSCLC samples. BMI1 expression was compared with known clinicopathological factors and biological markers in the NSCLC samples. There was a positive correlation between the expression of BMI1 and that of MMP9 in the SCC samples ($\chi^2=4.241$, P=0.039; Table I). Combined marker expression in the SCC samples was found to be significantly correlated with metastasis ($\chi^2=6.392$, P=0.014; Table II), but not with other clinical parameters in all lung cancer or AD cases.

BMI1 protein expression predicts OS in NSCLC. To determine whether BMI1 expression was an independent prognostic factor for OS in NSCLC, multivariate Cox regression analyses were performed. Among the immunological variables analyzed in this study, BMI1 expression was not associated with clinical outcome in the entire cohort. When analysis was stratified according to the major histological subtypes, the results suggested that BMI1 expression was an unfavorable prognostic factor in SCC (P=0.038; hazard ratio = 2.861; 95% confidence interval: 1.013-8.083; Table III and Fig. 2A). In AD, BMI1 tended to be a negative prognostic factor for long-term survival, although the difference was not statistically significant (P=0.203; hazard ratio = 1.633; 95% confidence interval: 0.762-3.501; Fig. 2B).

BMI1 and MMP9 protein expression predicts OS in SCC. Although MMP9 protein levels did not predict OS in NSCLC or its histological subtypes, the combination of high and low protein expression of BMI1 and MMP9 identified four

Table I. Correlation between BMI1 and MMP9 protein expression in squamous cell carcinoma.

BMI1 expression, n (%)	MMP9 expression, n (%)			χ^2	P-value
	High	Low	Total		
High	48 (60.8)	15 (19.0)	63 (79.8)	4.241	0.039 ^a
Low	8 (10.1)	8 (10.1)	16 (20.2)		
Total	56 (70.9)	23 (29.1)	79 (100)		

^aP<0.05 was considered to indicate statistically significant differences. High expression, immunohistochemical (IHC) scores ++, +++ and ++++; low expression, IHC scores - and +. BMI1, B-cell-specific Moloney murine leukemia virus integration site 1; MMP9, matrix metalloproteinase-9.

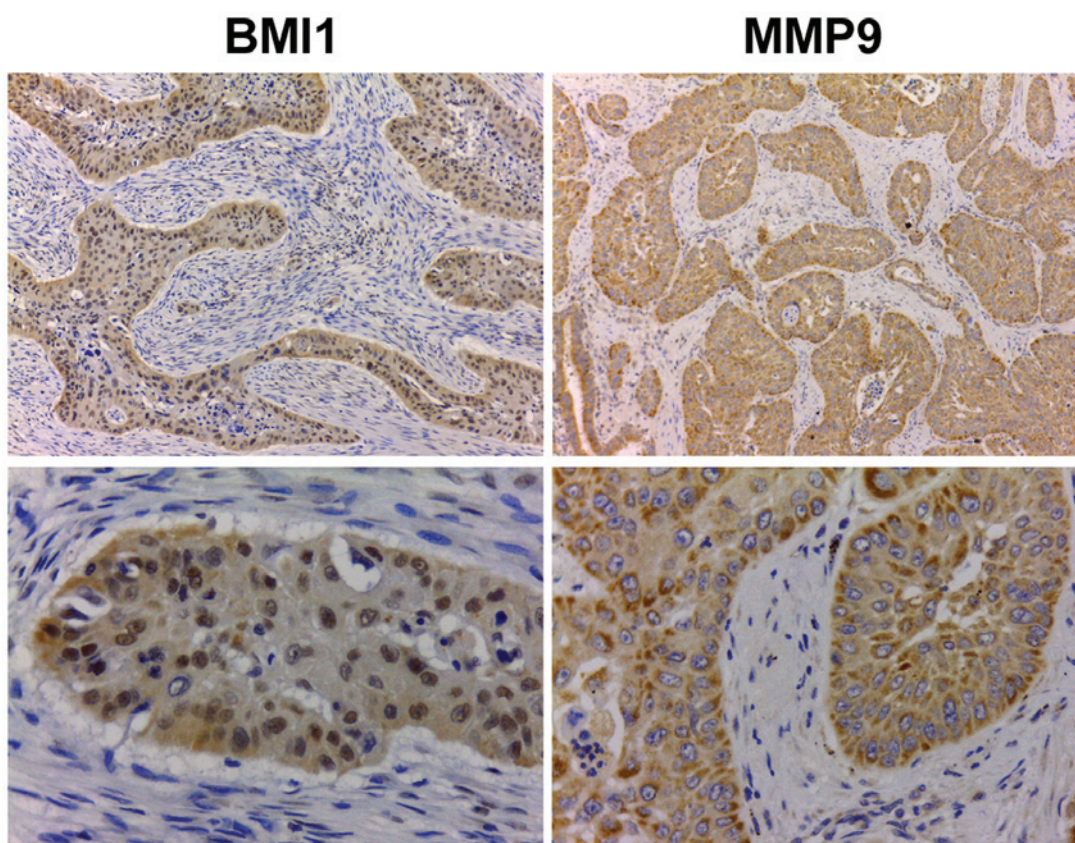


Figure 1. Representative immunohistochemical staining for BMI1 and MMP9 in an adenocarcinoma case. The sections were stained with BMI1- or MMP9-specific antibodies and counterstained with hematoxylin (upper panels: magnification, x100; lower panels: magnification, x400). BMI1, B-cell-specific Moloney murine leukemia virus integration site 1; MMP9, matrix metalloproteinase-9.

subgroups with distinct outcomes in SCC (Fig. 2C). The combination of high expression of BMI1 and MMP9 (Cluster B) was associated with shorter OS compared with low BMI1 and high MMP9 expression (Cluster A: High BMI1/low MMP9; low BMI1/high MMP9; and low BMI1/low MMP9). However, BMI1, MMP9 and combined marker expression were not significant prognostic factors in AD (Fig. 2D-F).

Discussion

Similar to other cancer types, lung cancer development is the result of a stepwise progression of malignant transformation of the normal respiratory epithelium (35). The classification

of lung cancer by Noguchi (35) identifies atypical adenomatous hyperplasia and cancer *in situ* as preinvasive neoplastic lung lesions that serve as precursors of invasive lung cancer through a progressive transformation into two types, characterized by survival outcome (35). Well-designed molecular biology investigations may be used for tumor classification, response prediction, assigning prognosis or even as therapeutic targets (36). SCC and AD share a range of similarities, but there are also differences (2,4,17). Therefore, identification of the roles of molecular markers in differential prediction of outcome is important in SCC and AD.

The BMI1 gene, which encodes a PcG protein, was first isolated as an oncogene that acts synergistically with c-Myc

Table II. Correlation of BMI1, MMP9 and combined marker expression levels with clinicopathological variables in squamous cell carcinoma.

Variables	All patients (n=79)	BMI1 expression		P-value	MMP9 expression		P-value	Combined marker expression		P-value
		High (n=63)	Low (n=16)		High (n=56)	Low (n=23)		High (n=47)	Low (n=32)	
Gender				0.960			0.275			0.143
Male	44	35	9		29	15		23	21	
Female	35	28	7		27	8		24	11	
Age (years)				0.455			0.760			0.769
<60	46	38	8		32	14		28	18	
≥60	33	25	8		24	9		19	14	
Smoking				0.468			0.450			0.116
Yes	43	33	10		32	11		29	14	
No	36	30	6		24	12		18	18	
Differentiation				0.679			0.621			0.465
Poor	31	24	7		21	10		20	11	
Moderate/high	48	39	9		35	13		27	21	
Pathological stage				0.082 ^a			0.312 ^a			0.239
I	41	35	6		26	15		23	18	
II	18	11	7		14	4		9	9	
III/IV	20	17	3		16	4		15	5	
Node/distant metastasis				0.248 ^a			0.111 ^a			0.014 ^b
Yes	25	22	3		21	4		20	5	
No	54	41	13		35	19		27	27	

^aDetermined by Fisher's exact test. ^bP<0.05 was considered to indicate statistically significant differences. BMI1, B-cell-specific Moloney murine leukemia virus integration site 1; MMP9, matrix metalloproteinase-9.

Table III. Univariate and multivariate analyses of overall survival for 79 patients with squamous cell lung carcinoma.

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Age (≥ 60 vs. < 60 years)	1.082 (0.992-1.179)	0.070		
Gender (male vs. female)	0.453 (0.134-1.414)	0.166		
Smoking status (yes vs. no)	1.260 (0.284-5.595)	0.180		
Differentiation (poor vs. moderate/high)	0.233 (0.675-1.287)	0.233		
Pathological stages (III/IV vs. II vs. I)	1.657 (0.889-3.089)	0.112		
Node/distant metastasis (yes vs. no)	2.551 (1.352-4.814)	0.004 ^a	17.163 (1.621-5.816)	0.001 ^a
BMI1 (positive vs. negative)	2.861 (1.013-8.083)	0.038 ^a		
MMP9 (positive vs. negative)	2.157 (0.092-4.888)	0.066		
Combined markers (cluster B vs. cluster A)	3.085 (1.466-6.491)	0.002 ^a	2.963 (1.142-7.637)	0.025 ^a

^aP<0.05 was considered statistically significant. HR, hazards ratio; CI, confidence interval.

Table IV. Summary of recently published studies on the correlation between BMI1 expression and clinicopathological factors or OS.

Study (Refs.)	Year	Cases (n)	Histology	Correlation	
				Clinicopathological factors	OS
Zhang <i>et al</i> (28)	2014	178	AD	pStage	Yes
Hu <i>et al</i> (25)	2012	114	NSCLC	Tumor size, differentiation, pT classification, pN classification, pStage	Yes
Huang <i>et al</i> (26)	2012	56	SCC	Tumor size, lymph node metastasis, distant metastasis	No analysis
Zhang <i>et al</i> (23)	2010	134	AD	pStage	Yes
Vrzalikova <i>et al</i> (22)	2008	179	NSCLC	pStage	No
Vonlanthen <i>et al</i> (19)	2001	48	NSCLC	No	No

pStage, pathological stage; BMI1, B-cell-specific Moloney murine leukemia virus integration site 1; OS, overall survival; AD, adenocarcinoma; NSCLC, non-small-cell lung cancer; SCC, squamous cell carcinoma.

in the oncogenesis of murine lymphomas (4,6). BMI1 functions as a transcriptional repressor of their target genes via an epigenetic mechanism (2). A number of studies have reported that BMI1 is upregulated in a variety of human malignancies, including lung (19), breast (11), gastric (13), hepatocellular (12) and esophageal (17) cancer, non-Hodgkin lymphoma (10) and cervical (15) cancer, suggesting that BMI1 is a potential oncogene. As a member of the PcG family, BMI1 plays a suppressive role, targeting cyclin-dependent kinase inhibitor 2A (p16^{INK4A}) and cyclin-dependent kinase 2-associated protein 2 (p14^{ARF}) (21,37,38), and promotes cell proliferation by suppressing the p16/retinoblastoma and/or p14^{ARF}/mouse double minute 2/p53 pathways (39,40). Furthermore, it has been reported that BMI1 may downregulate transcription of the tumor suppressor phosphatase and tensin homolog deleted on chromosome 10 (PTEN) via direct association with the PTEN gene locus (32). BMI1 upregulation also induces epithelial-to-mesenchymal transition and promotes human SCC occurrence, aggressiveness, invasion

and metastasis by downregulating E-cadherin and upregulating vimentin expression (26).

In this study, the expression of BMI1 in NSCLC samples was investigated. BMI1 was found to be significantly overexpressed in SCC compared with AD tissues. However, the present findings are contradictory to those of previous studies (22,25), which stated that BMI1 expression is not correlated with pathological type. Vrzalikova *et al* (22) reported a trend for higher BMI1 expression in SCC compared with AD, although the difference was not statistically significant. The discrepancies across studies may be due to the different immunohistochemistry protocols used, including antibody dilution, development time and positivity criteria applied, particularly the score used to discriminate positivity.

The present study demonstrated that high expression of BMI1 is associated with shorter OS and indicates that BMI1 expression confers an aggressive phenotype to human SCC. Current opinions on the significance of BMI1 expression in lung cancer

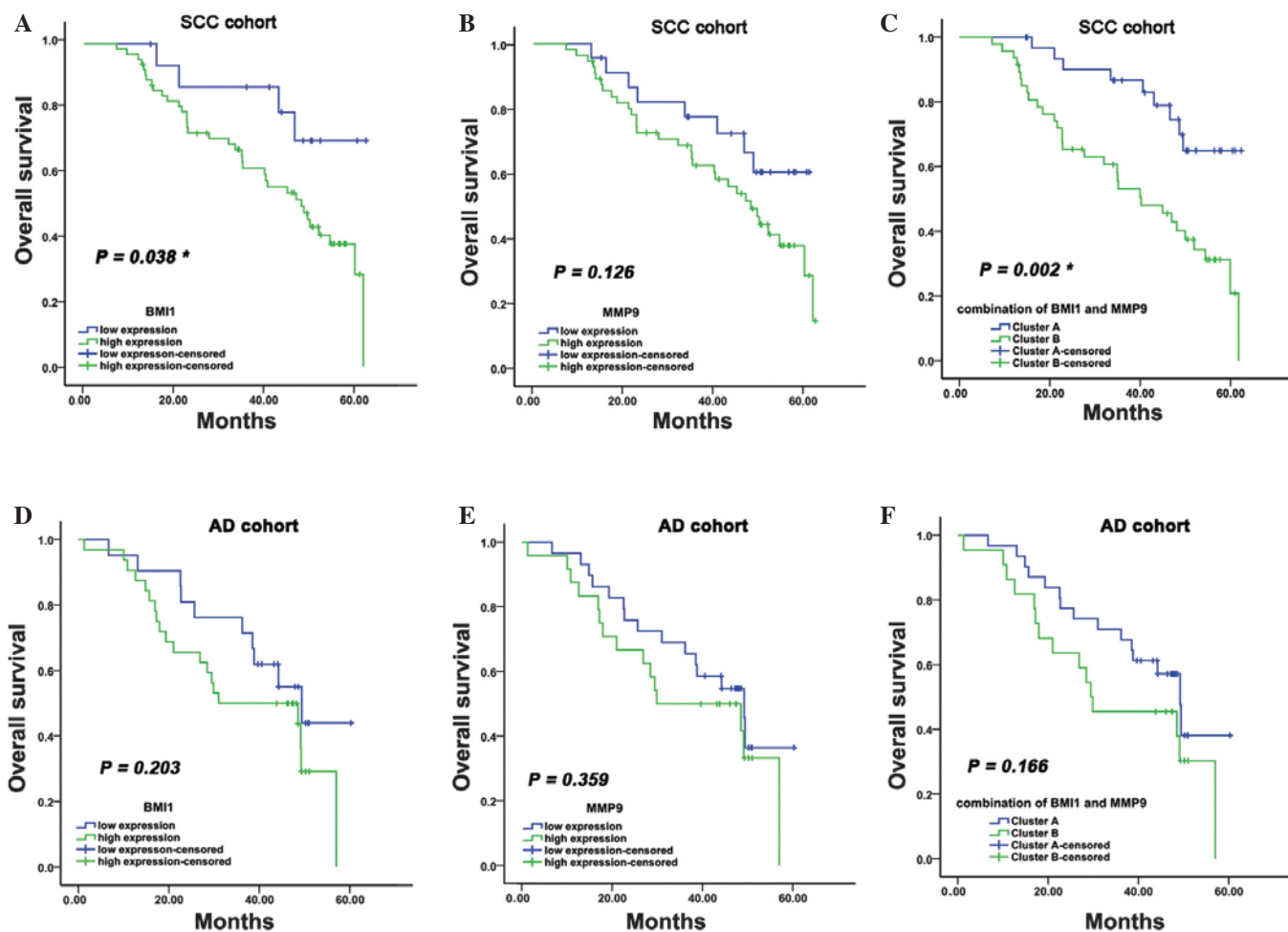


Figure 2. OS curves according to the level of BMI1, MMP9 and combined marker expression. (A and B) OS curves of 79 patients with SCC assessed by Kaplan-Meier analysis according to BMI1 and MMP9 expression. High BMI1 expression was significantly associated with shorter OS. (C) OS curves in SCC cases. OS for Cluster A (high BMI1/low MMP9, low BMI1/high MMP9 and low BMI1/low MMP9) was significantly shorter compared with that of Cluster B (high BMI1/high MMP9). (D and E) OS curves of 53 patients with AD assessed by Kaplan-Meier analysis according to BMI1 and MMP9 expression (F) OS curves in AD cases according to the combined marker expression as described in (C). * $P < 0.05$ was considered statistically significant. BMI1, B-cell-specific Moloney murine leukemia virus integration site 1; MMP9, matrix metalloproteinase-9; SCC, squamous cell carcinoma; AD, adenocarcinoma.

are diverse, and the effect of BMI1 expression on prognosis in patients with lung cancer remains controversial (Table IV). The present findings are similar to those of Huang *et al* (26), who reported that BMI1 induced epithelial-to-mesenchymal transition and enhanced lung SCC cell migration and invasiveness; however, they did not perform a correlation analysis between BMI1 and OS in their cohort of 56 SCC cases, but rather between BMI1 and nodal/distant metastasis. Notably, their cohort comprised a preponderance of cases with nodal/distant metastasis. The differences in patient clinicopathological factors between studies may explain this discrepancy.

Furthermore, the present study demonstrated that BMI1 expression is associated with MMP9 expression ($P = 0.039$). Of note, the combination of high BMI1 and high MMP9 protein levels was found to be significantly correlated with nodal/distant metastasis and predicted poor OS in SCC ($P = 0.002$). In human tumors, MMPs play a critical role in tissue remodeling during development in pathological processes, including inflammation, tissue repair, tumor invasion and metastasis (41,42). MMP expression has been reported to be low in the majority of benign lesions and is significantly increased in the majority of malignancies (43,44). Among MMPs, MMP9 is

of particular interest, as it is considered to be a tumor angiogenic factor that acts through VEGF (45). In NSCLC, MMP9 promotes the invasion of tumor cells *in vitro* and significantly promotes lung metastasis, mainly through specific induction of VEGFR-1 (29,30). Jiang *et al* (31) reported that BMI1 activates NF- κ B and subsequently upregulates MMP9 expression, leading to increased migration and invasion of glioma cells. In hepatocellular carcinoma, Li *et al* (32) reported that BMI1 overexpression contributed to invasion and metastasis by increasing MMP9 expression via the PTEN/PI3K/Akt pathway. However, the BMI1-MMP9 interaction in NSCLC invasion and metastasis is poorly understood. The present results indicate that the combination of high BMI1 and high MMP9 expression, rather than high BMI1 expression alone, is an independent prognostic indicator of OS in SCC. We hypothesize that there is a BMI1-MMP9 signaling pathway in SCC and that its activity promotes SCC progression and metastasis, although current models do not fully explain the complex interactions between BMI1 and MMP9.

In conclusion, BMI1 is upregulated in lung SCC tissues compared with AD tissues, and BMI1 expression is associated with adverse prognosis of SCC. BMI1 expression is associated

with that of MMP9, and the combination of high BMI1 and high MMP9 protein levels are significantly correlated with nodal/distant metastasis. Additionally, the combined marker expression is an independent prognostic indicator of OS in SCC. The potential mechanism underlying the involvement of BMI1 and MMP9 in tissue-specific SCC progression requires further elucidation by future studies.

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