B-cell specific Moloney leukemia virus insert site 1 and peptidyl arginine deiminase IV positively regulate carcinogenesis and progression of esophageal squamous cell carcinoma

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Abstract. High expression of B-cell specific Moloney leukemia virus insert site 1 (Bmi-1) and peptidyl arginine deiminase IV (PADI4) is associated with esophageal carcinoma. However, few studies have investigated the association between the Bmi-1 and PADI4 genes. The aim of the present study was to evaluate the expression of Bmi-1 and PADI4 and identify the association between the Bmi-1 and PADI4 genes in esophageal squamous cell carcinoma (ESCC) tissues. Bmi-1 and PADI4 gene expression levels were measured using immunohistochemistry, western blotting and reverse transcription-quantitative polymerase chain reaction in ESCC tissues from 86 patients who had not received pre-operative chemoradiation. The results revealed that the Bmi-1 and PADI4 genes had increased expression in carcinoma tissues compared with pericarcinous tissue (P<0.05). Bmi-1 gene expression was revealed to be associated with differentiation degree, clinical stage and lymph node metastasis (P<0.05), but had no association with gender, age or depth of invasion (P>0.05). The expression of PADI4 was associated with clinical stage, depth of invasion and lymph node metastasis (P<0.05), but was not associated with gender, age or differentiation degree (P>0.05). In addition, there was a positive association between Bmi-1 and PADI4 gene expression in ESCC (P<0.05). These results indicated that Bmi-1 and PADI4 positively regulate carcinogenesis and progression of ESCC.

Introduction

Esophageal cancer is the eighth most common type of cancer in the world, making it a serious threat to human health (1). There are ~240,000 new esophageal cancer cases in China every year (2). Esophageal squamous cell carcinoma (ESCC) is the major histological type of esophageal cancer. ESCC is a highly aggressive malignancy due to late diagnosis, rapid progression and poor prognosis of survival. Therefore, the mortality rate of esophageal cancer is similar to the incidence of esophageal cancer (3,4). There have been numerous improvements in surgery, radiotherapy and chemotherapy; however, the 5-year overall survival rate remains poor due to diagnosis of the disease at an advanced stage (5). It is important for ideal markers to be identified as these may help in early diagnosis of the disease.

The B-cell specific Moloney leukemia virus insert site 1 (Bmi-1) gene, a member of the polycomb group of proteins, was identified in 1999 and originally isolated as an oncogene in the generation of mouse pre-B-cell lymphomas (6). As a transcriptional repressor through chromatin modification, Bmi-1 is involved in axial patterning, cell cycle regulation, hematopoiesis and senescence (7,8). Overexpression of Bmi-1 has been observed in a variety of human cancers, including gastric (9), breast (10), colorectal (11) and head and neck cancers (12), and it was also observed that expression of Bmi-1 is associated with the development of tumors (13,14). It was reported that overexpression of Bmi-1 in primary human cancer cells may cause downregulation of the INK4a-ARF locus and affect the pathway of p14ARF-Mdm2-p53 (15). It has been hypothesized that Bmi-1 is an important inhibitor of the p53 pathway (13,15).

Peptidyl arginine deiminase IV (PADI4 or PAD4) is a post-translational modification enzyme that converts arginine residues at histone tails to citrulline, in the presence of Ca2+ (16). The expression of PADI4 has been detected in...
human CD34+ stem/progenitor cells (17), and it is involved in the regulation of hematopoietic progenitor proliferation (18). Our previous study detected the overexpression of PADI4 in various malignancies, including lung adenocarcinoma, hepatocellular cancer, breast cancer and metastatic cancer (19). Tanikawa et al reported that citrullination of H4R3 by PADI4 is associated with the p53 pathway (20).

In the present study, the expression levels and clinical significance of Bmi-1 and PADI4 were investigated in esophageal cancer tissues and pericarcinomatous tissues in order to observe differences in the expression of Bmi-1 and PADI4.

**Materials and methods**

**ESCC patient sample preparation.** The samples of esophageal cancer tissues and adjacent noncancerous mucosal tissues were obtained from 26 patients who had received surgical treatment between January 2014 and December 2015 in the Department of Chest Surgery, Qianfoshan Hospital of Shandong University (Shandong, China). Normal tissues located 5 cm away from the tumor edge were collected during surgery. Tissue microarrays containing 120 esophageal tissue sections were commercially obtained from Chaoying Bioscience (Shanxi, China). The slides contained ESCC tissues (n=60) and adjacent normal tissues. Section thickness was ~3 µm. Firstly, the sections were stained with hematoxylin and eosin (Boster, Co., Ltd., Beijing, China) within each tissue core. All patients had not received radiotherapy or chemotherapy prior to surgery. Postoperative pathologic results of the esophageal cancer biopsies showed they were all squamous cell carcinomas. Patient information, including gender, age and clinicopathological characteristics, was obtained from the medical records or the manufacturer. The enrolled patients included 61 males and 25 females, and the median age was 58 years (range, 36-76). According to the World Health Organization standard pathology classification, the tumors of 9 patients (10.5%) were diagnosed as well-differentiated tumors, the tumors of 47 patients (54.7%) were diagnosed as moderately differentiated tumors, and the tumors of 30 patients (34.9%) were diagnosed as poorly differentiated tumors. In these cases, 70 patients had no lymph node metastasis and 16 patients had lymph node metastasis. All patients provided informed consent prior to specimen acquisition and the present study was approved by the Research Ethics Committee of the Qianfoshan Hospital Affiliated to Shandong University.

**Immunohistochemical staining.** Tissue samples were soaked in 10% neutral buffered formalin, embedded in conventional paraffin, and the section thickness was ~3 µm. Following deparaffinization, the specimens were hydrated and incubated with an epitope retrieval solution (Boster, Co., Ltd.; pH 6.0) in a microwave (temperature controlled at 95-100°C) for 20 min. The slices were then cooled to room temperature and incubated with 0.3% H2O2 for 10 min at room temperature to inactivate endogenous peroxidase, and then rinsed with PBS. The specimens were then incubated with rabbit polyclonal antibody for Bmi-1 (cat. no., ab85688; dilution, 1:8,000; Abcam) and mouse monoclonal antibody for PADI4 (cat. no., ab128086; dilution, 1:500; Abcam) at 4°C overnight. Specimens were then incubated with ready-to-use secondary antibody EliVision™ plus kit (cat. no., KIT-9901; Maixin-Bio; Lab Vision, Kalamazoo, USA), according to the manufacturer's protocol. The specimens were then washed using PBS, and dianaminobenzidine (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) chromogenic reagent was added. Termination of the chromogenic reaction was achieved with water. Following counterstaining with hematoxylin, specimens were dehydrated, mounted and observed under a Nikon 5i0 fluorescence microscope, magnification, x200 (Nikon Corporation, Tokyo, Japan).

**Interpretation of the results.** The immunohistochemical specimens were evaluated by German semi-quantitative statistical methods. Briefly, slices were observed under an optical microscope (magnification, x200) and evaluated using positive staining intensity and percentage of positive staining by two pathologists. Positive staining intensity was rated as follows: 0, No staining; 1, light yellow; 2, yellow; 3, dark yellow. The extent of stained cells was ranked as follows: 0, 0-5%; 1, 5-25%; 2, 25-50%; 3, 50-75%; 4, 75-100%. The final score was determined by multiplying the staining intensity scores with the extent of positivity scores of cells: 0-2, negative (-); 3-5, weak (+); 6-8, moderate (++); and 9-12, strong (+++) (21).

**Western blot analysis.** Human esophageal cancer tissues were homogenized and centrifuged at 12,000 x g for 30 min at 4°C. The protein concentrations were determined using the bicinchoninic acid protein assay kit (Boster Biological Technology, Pleasanton, CA, USA). Total protein (30 µg) was separated by 10% SDS-PAGE, transferred to a polyvinylidene fluoride membrane and blocked in 5% milk. The membranes were incubated with rabbit polyclonal antibody for Bmi-1 (cat. no., ab85688, dilution, 1:8,000; Abcam) and mouse monoclonal antibody for PADI4 (cat. no., ab128086, dilution; 1:2,000; Abcam) or mouse antibody for GAPDH (cat. no., AF0006; dilution, 1:6,000; Beyotime Institute of Biotechnology, Haimen, China) at 4°C overnight. The Goat anti-Mouse secondary antibody (cat. no., A0258; dilution, 1:8,000; Beyotime Institute of Biotechnology) and Goat anti-Rabbit secondary antibody (cat. no., A0239; dilution, 1:8,000; Beyotime Institute of Biotechnology) was incubated with the membrane for 1 h at room temperature in TBS with Tween-20. Finally, the immunoreactive protein bands were visualized with Immobilon™ Western Chemiluminescent HRP Substrate (EMD Millipore, Billerica, MA, USA).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from frozen specimens using the E.Z.N.A.® total RNA kit II (Omega Bio-Tek, Inc., Norcross, GA, USA), according to the manufacturer's protocol. Total RNA (1 µg) was then used to perform reverse transcription for first-strand cDNA using the revert aid first strand cDNA synthesis kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Subsequently, qPCR was performed in triplicate on the ABI VIIA7 real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with SuperReal PreMix Plus (SYBR-Green; Tiangen Biotech Co., Ltd., Beijing, China) with primers that amplified a specific single product by melt curve. Primer sequences for Bmi-1, PADI4 and β-actin were as follows: Bmi-1 forward, 5′-CCACCTGATGTGTGTGCT
TTG-3' and reverse, 5'-TTC AGT AGT GGT CTG GTC TTGT-3'; PADI4 forward, 5'-GGGGTGGGTGAGTTATGG-3' and reverse, 5'-GCCGGTGGTAGTATGGAGC-3'; and β-actin forward, 5'-GACCACACCTCTCAACTGAG-3' and reverse, 5'-GCTACCCCTCCTGATGGC-3'.

All reactions were done in a 20 µl reaction volume. Subsequent to pre-denaturing, PCR was performed at 95°C for 15 min, followed by 40 cycles at 95°C for 10 sec and 62°C for 32 sec. Gene expression was analyzed with the comparative threshold cycle (Cq) method following normalization to the reference gene β-actin. ∆∆Cq was used to calculate the relative amount of the transcripts in the esophageal cancer samples and the control group, which were normalized to the endogenous control (22).

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\Delta\Delta Cq = \Delta Cq (\text{esophageal cancer}) - \Delta Cq (\text{control})
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The fold change for each esophageal cancer sample relative to the control sample=2^(-ΔΔCq). When the expression showed a 2-fold decrease or increase compared with normal counterpart tissue, it was considered as an altered expression.

Statistical analysis. The correlation between Bmi-1 and PADI4 mRNA expression levels was analyzed by Pearson's coefficient test. The experimental data of protein expression was statistically analyzed with χ² test and Fisher's exact test. All statistical analyses were performed using the SPSS 21.0 software package (IBM SPSS, Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Immunodetection of Bmi-1 and PADI4 in ESCC tissues. The expression rates of Bmi-1 in ESCC tissue and normal mucosa were 73.3 and 30.2%, respectively. The expression rates of PADI4 in ESCC tissue and normal mucosa were 68.6 and 37.2%, respectively. The differences in the expression of Bmi-1 and PADI4 were statistically significant between esophageal cancer tissue and normal esophageal mucosa (P<0.05; Tables I and II).

Association between the expression of Bmi-1 and PADI4 with clinical pathological parameters. The importance of Bmi-1 and PADI4 in ESCC was evaluated by correlating its expression level with clinicopathological features. Several of the analyzed clinicopathological features exhibited significant associations with the expression levels (Table III). The results revealed that the expression of Bmi-1 was associated with differentiation degree, clinical stage and lymph node metastasis (P<0.05), but not with patient gender, age and depth of invasion (P>0.05). The expression of PADI4 was associated with clinical stage, depth of invasion and lymph node metastasis (P<0.05), but not with patient gender, age and differentiation degree (P>0.05).

Quantifying Bmi-1 and PADI4 expression levels by western blot analysis and RT-qPCR. Bmi-1 and PADI4 protein levels were quantified by western blot analysis. The protein was detected in ESCC and corresponding para-carcinoma tissues. Compared with para-carcinoma tissues, the expression levels of Bmi-1 and PADI4 were significantly increased in ESCC, with statistically significant differences (P=0.014 and 0.021, respectively; Fig. 2). Transcription of Bmi-1 and PADI4 was quantified by RT-qPCR. The results revealed that Bmi-1 mRNA was overexpressed in 17 of 26 (65.38%) esophageal cancer tissues (>2-fold), and PADI4 mRNA was overexpressed in 14 of 26 (53.85%; >2-fold). As shown in Fig. 3 and Table IV, the ESCC tissues exhibited an increased level of Bmi-1 and PADI4 mRNA compared with the para-carcinoma tissues.
Correlation between Bmi-1 expression and PADI4 expression in ESCC tissues. Correlation analysis was performed to test the correlation between the expression of Bmi-1 mRNA and PADI4 mRNA. Bmi-1 overexpression was positively associated with PADI4 expression (r=0.534; P=0.005). In addition, according to the results of immunohistochemical studies, the correlation coefficients indicated that there were significant positive correlations between Bmi-1 and PADI4 genes (r=0.214; P=0.047; Table V).

Discussion

It is well known that ESCC, a common malignant disease, is prone to invade adjacent regions and metastasize to lymph nodes or distant organs. At the point of diagnosis, metastasis...
has already occurred in >50% of patients with ESCC, with no chance of resection (23), which largely explains the poor prognosis of ESCC (5). Therefore, diagnostic markers of early ESCC may be useful to improve prognosis, and for selecting treatments properly. The purpose of the present study was to investigate the expression and clinicopathological roles of Bmi-1 and PADI4 in ESCC.

Mammalian polycomb group (PcG) protein complexes are generally classified as polycomb repressive complexes 1 or 2 (PRC1 or PRC2). Alterations in PcG expression have been observed in human tumors (24,25). Bmi-1, a PRC1 that regulates proliferation and senescence in mammalian cells, plays an important role in the self-renewal of stem cells. Overexpression of Bmi-1 has been observed in several human cancers and its overexpression is often associated with poor prognosis in gastric cancer, bladder cancer and esophageal squamous cell carcinoma (2,24‑26). It was reported that Bmi-1, as a proto-oncogene, plays an important role in the invasion and metastasis of neoplasms. Overexpression of Bmi-1 in esophageal cancer has been reported (25). The depth of invasion, clinical stage, lymph node metastasis status and lower survival rate have all been identified as associated with Bmi-1 overexpression (2,25). In the present study, it was revealed that Bmi-1 plays an important role in ESCC progression. It was demonstrated that Bmi-1 expression is significantly upregulated (P<0.05) in ESCC compared with para-carcinoma tissue samples. Bmi-1, B-cell specific Moloney leukemia virus insert site 1; PADI4, peptidyl arginine deiminase IV; T, primary esophageal tumors; N, normal esophageal tissue.

Genome-wide analysis has demonstrated that PADI4 functions as an activator of gene expression by citrullination of transcription factors (27). Previous studies demonstrated that PADI4 is part of a transcriptional network that regulates pluripotency (28‑31). It has been suggested that PADI4 may target different histone arginines (32,33). Kolodziej et al
previously identified PADI4 as a novel interaction partner of T-cell acute lymphocytic leukemia protein 1 (Tal1), which is a critical regulator of hematopoietic gene expression and may act as an oncogene if aberrantly expressed, and identified a large number of genes that are co-regulated by PADI4 and Tal1 (31). Overexpression of PADI4 is often observed in growth of tumors, and the inhibitor Cl-amidine reduces growth of a subset of cell lines (34). Chang et al confirmed that the expression level of PADI4 was positively associated with the pathological classification of ESCC. The previous study also identified that apoptosis corresponded to the expression of PADI4 in cultured EC cells that were treated with dichloroacetate (35). In the present study, the expression of PADI4 was significantly increased in ESCC tissues compared with the corresponding noncancerous mucosal tissues. In addition, it was also observed that positive expression of PADI4 was correlated with the corresponding noncancerous mucosal tissues. In addition, it was also observed that positive expression of PADI4 was correlated with the depth of cancer invasion (T classification), clinical stage and lymph node metastasis (N classification), with statistically significant differences (P<0.05). This indicated that PADI4 protein is associated with esophageal carcinogenesis, progression and metastasis, and that PADI4 may play a crucial role in ESCC.

The present study demonstrated that Bmi-1 and PADI4 are expressed abnormally in ESCC. The expression levels of Bmi-1 and PADI4 are associated with the carcinogenesis and progression of human tumors. However, the expression of Bmi-1 is not associated with depth of invasion (P>0.05). No association was observed between PADI4 expression and differentiation degree (P>0.05). These results may be due to the small size of patient cohort, and additional studies using larger samples are required to extend the present findings. The present study also identified a positive correlation between Bmi-1 and PADI4 expression at mRNA (r=0.534; P=0.005) and protein levels (r=0.214; P=0.047). However, it remains unknown how Bmi-1 interaction with PADI4 affects carcinogenesis, progression and metastasis in ESCC.

The p14ARF-MDM2-p53 pathway, commonly referred to as the p53 pathway, is an important pathway in the development and progression of numerous human malignancies (36). The p53 pathway is usually inactivated by TP53 mutation, amplification of MDM2 or p14ARF deletion in a number of human cancers (37,38). Previous studies have demonstrated that Bmi-1 is a potent repressor of the p14ARF-MDM2-p53 pathway (13,39,40). Inactivation of the p53 pathway by Bmi-1 has been identified in lymphomagenesis and oncogenesis in human non-small cell lung cancer (15,37,41). Yao et al reported that there is an inverse correlation between the expression of Bmi-1 and p14ARF, and thereby dysfunction of the p53 growth regulatory pathway during the development of gastric cardia adenocarcinoma (42). In addition, a number of studies proposed that PADI4 plays an important role in various cellular processes, including proliferation, the cell cycle and apoptosis (27,30-33). PADI4 overexpression has also been considered to reduce the expression of p53-targeted genes, resulting in the disruption of cellular apoptosis and of the normal cell cycle (35,43,44). Previous studies have confirmed that PADI4 disrupts the apoptotic process via citrullination of histone H3, which acts on the promoter of p53 target genes (44,45). Cui et al hypothesized that PADI4 may regulate migration, invasion and apoptosis in A2780 cells with wild-type p53 and in p53-null SKOV3 cells, and that PADI4 may be associated with the p53 gene (29). Apoptosis PCR array analysis demonstrated that PADI4 overexpression induced the decreased expression of the Fas ligand gene,
which is one of the important genes downstream of p53. Additional studies proposed that TNF receptor superfamily member (TNFRSF) 9, Bcl-2 like protein 2, TNFRSF11B and Bcl-2 antagonist/killer 1 may perform important roles in the mechanism of action of PADI4, which is associated with ovarian tumorigenesis (29). The mechanism may be associated with the p53 gene (46,47).

According to the present results, it was speculated that Bmi-1 and PADI4 may be involved in the associated signaling molecules of the p53 pathway, which mediate the regulation of esophageal cancer. It was hypothesized that overexpression of Bmi-1 may inhibit the activation of p14ARF and the function of the p53 pathway. It leads to tumor formation by regulating the expression of PADI4. Bmi-1, which is associated with PADI4 by the p14ARF-MDM2-p53 pathway, positively regulated the carcinogenesis and progression of ESCC.

To the best of our knowledge, this is the first study to investigate the association between Bmi-1 and PADI4 expression in ESCC. The expression of Bmi-1 and PADI4 was associated with esophageal cancer progression. The advanced stages of ESCC are more likely to express increased levels of Bmi-1 and PADI4. The present study detected that the associated gene expression and the mechanism may be associated with p53; however, the detailed mechanisms and how they are regulated in esophageal cancers were not investigated.

In conclusion, the present study demonstrated that the expression of Bmi-1 and PADI4 was associated with the carcinogenesis and progression of ESCC. Therefore, Bmi-1 and PADI4 may be used as prognostic markers in ESCC. In addition, a positive association was observed between Bmi-1 and PADI4, and this mechanism may be associated with the p53 pathway.

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References


