# Aberrant Expression of EZH2 Is Associated with Pathological Findings and P53 Alteration

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**Abstract.** *Aim: Enhancer of zeste homolog-2 (EZH2) and B lymphoma Mo-MLV insertion region-1 homolog (BMI1) are members of the polycomb group of proteins, which function as transcriptional repressors through chromatin modification. EZH2 forms part of the polycomb repressive complex (PRC)-2, while BMI1 is a component of PRC1. Previous studies have shown that EZH2 is highly expressed in various type of cancers. Expression of EZH2 is reported to be regulated by the P53-E2F/retinoblastoma (RB)-related pathway, and a correlation between P53 mutation and EZH2 expression was recently found in breast cancer. Here, we examined the relationship between P53 and EZH2 in oral squamous cell carcinoma (OSCC). Materials and Methods: Using immunohistochemistry, we investigated the expression of EZH2 and BMI1 in 99 surgically-resected OSCC and 34 epithelial dysplasia samples. We analyzed associations between aberrant expression of EZH2 and BMI1, and clinicopathological findings and patient outcome. P53 expression was also examined and analyzed in relation to EZH2 and BMI1 expression. Results: EZH2 and BMI1 protein were up-regulated in OSCC tissues compared with epithelial dysplasia and normal epithelium. Aberrant EZH2 and BMI1 protein expression was observed in 32 and 59 of the 99 OSCC samples, respectively. Aberrant EZH2 and BMI1 expression was significantly associated with mode of invasion, but not with lymph node metastasis or survival rate. Aberrant EZH2 expression was associated with P53 alteration in OSCC tissue. Expression of EZH2 mRNA in SAS/neo cells, which have wild-type P53, was significantly lower than in SAS/mp53 cells that have a mutant P53 gene. Conclusion: P53 alteration may be involved in dysregulated EZH2 expression, and aberrant expression of EZH2 may play a role in carcinogenesis of OSCC.*

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Oral squamous cell carcinoma (OSCC) is the most common malignancy of the upper aerodigestive tract (1). Dissemination of OSCC through lymphatic routes may occur in the early course of the disease. Enhancer of zeste homolog-2 (EZH2) and B lymphoma Mo-MLV insertion region-1 homolog (BMI1) are members of the polycomb group (PcG) of proteins. PcG genes have been identified in *Drosophila melanogaster* as repressors of homeobox genes throughout development (2). The polycomb repressive complex 1 (PRC1), which maintains transcriptional repression, is composed of BMI1, MEL18, Ring finger protein-1 (RING1), Human Polyhomeotic (HPH), and Human Polycomb (HPC) PcG proteins. The PRC2 initiation complex is composed of EZH2, Embryonic ectodermal development (EED), Yin Yong 1 (YY1), and Suppressor of zoste 12 homolog (SUZ12) PcG proteins (3).

Previous studies have shown that EZH2 is highly expressed in prostate (4) and breast cancers (5), and that its expression is associated with a poor outcome in both conditions (6, 7). This association of increased expression of EZH2 and poor outcome has also been reported for other types of cancer, including colorectal (8), stomach (9), and OSCC (10). EZH2 is implicated in cell proliferation and cell-cycle regulation. Inhibition of EZH2 expression by small-interfering RNA (siRNA) inhibits cell proliferation, resulting in cell-cycle arrest and reduced cancer cell invasiveness (4, 11-14). Overexpression of EZH2 promotes cell proliferation, anchorage-independent growth, and cell invasion *in vitro* (5, 13, 15). Although the precise mechanism through which EZH2 contributes to these malignant phenotypes has not been fully elucidated, EZH2 is suspected of mediating the repression of tumor suppressor genes such as DOC-2/DAB-2 interacting protein (DAB2IP) (16), E-cadherin (17), and Runt-related transcription factor-3 (RUNX3) (18).

BMI1 controls the cell cycle and self-renewal of tissue stem cells (19). It can influence the central tumor

suppressors Rb and P53 by suppressing the p16 INK4a/p19 ARF locus (20). BMI1 is overexpressed in OSCC cells compared with normal mucosa, and is thought to influence cell proliferation and survival in oral carcinogenesis (21). Overexpression of BMI1 correlates with poor prognosis in the non-keratinising type of nasopharyngeal carcinoma (22), in breast and hepatocellular carcinoma (23, 24), as well as in nervous system tumors such as oligodendrogliomas and medulloblastomas (25, 26). A correlation of BMI1 with tumor stage has also been reported in non-small-cell lung cancer (27).

Mutation of the *P53* gene is a frequent early event in the carcinogenesis of squamous cell carcinoma (28-33). A recent study showed a correlation between P53 mutation and EZH2 expression in breast cancer tissue (37). The expression of EZH2 has been reported to be regulated by the p53-RB-E2F pathway (11, 12), MYC (34), microRNA-26a (34, 35), and microRNA-101 (36).

In this study, we aimed to determine the stage at which EZH2 and BMI1 expression are altered during epithelial carcinogenesis. To this end, the expression of the PcG genes EZH2 and BMI1 was analyzed in OSCC lesions, normal epithelium, and epithelial dysplasia lesions. We also sought to determine whether EZH2 and BMI1 expression relates to P53 expression.

## Materials and Methods

*Human tissues*. Ninety-nine consecutive patients with OSCC (50 men, 49 women; median age 65.3 years; age range 25-84 years) who underwent surgical resection with curative intent between January 2007 and December 2011 at the Showa University Dental Hospital, were enrolled in this study. Thirty-four patients with leukoplakia also underwent surgical resection in the same period. Clinicopathological findings were assessed based on the International Union Against Cancer TNM staging system (38). There were 32 stage I cases, 31 stage II cases, 24 stage III cases, and 12 stage IV cases. According to the histological grade criteria of the World Health Organization(39), 53 (53.5%) cases was well-differentiated, 39 (39.3%) moderately-differentiated, and seven (7%) poorly differentiated. The histological mode of invasion was classified according to the method of Anneroth (40). Follow-up data were available for all patients. The median follow-up period for the disease-free surviving patients was 792.2 months (range 30-1800 months).

Dysplasia of squamous epithelium occurs on a spectrum and can be graded as low, intermediate, or high according to the World Health Organization (WHO) 2005 classification (39). It is defined by the presence of certain architectural and cytological features of the epithelium. Architectural disturbances of squamous epithelium include irregular epithelial stratification, loss of cellular polarity, increased mitotic figures, dyskeratosis, and presence of keratin pearls within rete pegs. Cytological atypia is manifested by nuclear pleomorphism, anisocytosis, increased nuclear-cytoplasmic ratio, and increased number and size of nucleoli. In this study, 12 cases were classified as mild dysplasia, 11 as moderate, and 11 as severe.

*Immunohistochemistry*. All resected specimens were fixed in either 10% formalin or methanol, and embedded in paraffin. Sections (4-μm-thick) were cut from the paraffin-embedded block that contained the most representative area of the tumor and were used for immunohistochemical staining. The sections were deparaffinized in xylene, dehydrated in a graded series of ethanol, and immersed for 15 min in methanol containing 0.3% hydrogen peroxide to inhibit endogenous peroxidase activity. For antigen retrieval, the slides were autoclaved in citrate buffer (pH 6.0) at 121˚C for 15 min and allowed to cool to room temperature. The slides were incubated with primary antibodies overnight at 4˚C, washed three times with PBS, and incubated with Simple StainTM MAX-PO (Multi; Nichirei, Tokyo, Japan) for 1 h at room temperature. The sections were visualized using Envision HRP/ Kit (DAB; Dako, Kyoto, Japan) and counterstained with hematoxylin. The antibodies used in this study were to EZH2 (mouse monoclonal, clone 11, 1:2500; BD Biosciences, San Jose, California, USA), anti-BMI1 (mouse monoclonal, clone F6, 1:100; Upstate Biotechnology, Lake Placid, New York, USA) and P53 (mouse polyclonal, clone PAb1801, 1:1000; EMD Millipore, Billerica, Massachusetts, USA). Negative controls were performed by omitting the primary antibodies.

To assess EZH2 and BMI1 protein expression, a minimum of 500 cells in each tumor and adjacent non-neoplastic oral epithelium were counted, and the percentage of cells with positive nuclear staining was recorded for each. OSCC tissue in which 5% or more of the cancer cells were stained positive was considered positive for aberrant expression of the respective protein. Each case was then classified into a high-expression (≥median) or low-expression (<median) group. P53 staining was considered positive when >10% of the cells exhibited strong nuclear staining.

*Cell cultures*. Human OSCC cell lines SAS/neo and SAS/mp53 (P53 mutant; codon 248 mutated from Arg to Trp) were purchased from RIKEN BRC (Tsukuba, Japan). Cells were cultured at 37˚C in Dulbecco's Modified Eagle's Medium (DMEM) (Wako, Osaka, Japan) supplemented with 10% Fetal bovine serum (FBS) (PAA, Pasching, Austria) and penicillin/streptomycin (Life Technologies, Carlsbad, California, USA), under a 5% $CO_2$ atmosphere.

*RNA isolation and real-time (RT PCR)*. SAS/neo cells and SAS/mp53 cells were seeded in 6-well culture plates at a density of $1\times10^5$ cells per well, and after incubation at 37˚C for 48 h, total RNA was isolated using TRIzol® Reagent (Life Technologies). cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, California, USA) according to the manufacturer's instructions. Real-time PCR was performed in a MYiQ2 (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad). Expression of *EZH2* and *BMI1* was normalized to that of glyceraldehydes-3-phosphate dehydrogenase (GAPDH), and the relative expression level was calculated. The following PCR primer pairs were used: *EZH2* 5'-GCCAGACTGGGAAGAAATCTG-3' (forward) and 5'-TGTG TTGGAAAATCCAAGTCA-3' (reverse); *BMI1* 5'-CAGCAATGA CTGTGATGCACT-3' (forward) and 5'-GCCCAATGCTTATGTCC ACT-3' (reverse); and *GAPDH* 5'-ACCACAGTCCATGCCATCAC-3' (forward) and 5'-TCCACCACCCTGTTGCTGTA-3' (reverse).

*Immunoblot analysis*. Immunoblot analysis was performed using standard procedures. Briefly, cells were lysed with TritonX-100 lysis buffer (50 mM Tris pH 8.0, 300 mM NaCl, 0.5% TritonX-100, 5 mM EDTA, 1 mM sodium o-vanadate) supplemented with

Complete MiniTM protease inhibitor tablets (Roche Diagnostics, Mannheim, Germany). Frozen tissue was porphyrized using a POLYTRON System RT 2500E (Central Scientific Commerce, Inc., Tokyo, Japan). Protein concentration was measured using the Quick Start Protein Assay (Bio-Rad Laboratories). Whole cell lysates (25 μg protein) were separated with a 10-20% Ready Gel (Bio-Rad) and transferred to a PVDF membrane using iBLOT Transfer Stacks (Life Technologies). The membrane was blocked with bovine serum albumin/PBS and incubated with primary antibody for 90 min at room temperature. The blots were visualized using Western Lightning Plus (Perkin-Elmer, Waltham, MA, USA).

*Statistical analysis*. The association between aberrant EZH2 and BMI1 expression with clinicopathological features and the expression of other proteins was evaluated using Fisher's exact test. Differences in the levels of expression of EZH2 and BMI1 in cell lines were analyzed using Student's *t*-test. Cumulative survival curves were drawn by the Kaplan–Meier method, and differences between the curves were analyzed using the log-rank test. All *p*-values were two-sided and a value of $p<0.05$ was considered significant. All analyses were performed using GenePattern 3.5 for Windows (BROAD Institute Inc., Cambridge, MA, USA).

Spearman's rank correlation was used to determine whether a correlation between EZH2 and P53, and BMI1 and P53 expression was positive or negative. The expression of these proteins and clinicopathological parameters was examined using Fisher's exact test. Disease-free survival was calculated using the Kaplan–Meier method, and a comparison between groups was performed using the log-rank test. Statistical significance was set at $p<0.05$.

Table I. *Correlation between the clinicopathological features and expression of B lymphoma Mo-MLV insertion region 1 homolog (BMI1) and Enhancer of zeste homolog-2 (EZH2) protein.*

| | Aberrant BMI1 expression | | | Aberrant EZH2 expression | | |
|---|---|---|---|---|---|---|
| | Positive (n=59) | Negative (n=40) | *p*-Value | Positive (n=32) | Negative (n=67) | *p*-Value |
| Age | | | | | | |
| ≥60 years | 45 | 28 | 0.495 | 26 | 47 | 0.330 |
| <60 years | 14 | 12 | | 6 | 20 | |
| Gender | | | | | | |
| Male | 29 | 21 | 1 | 12 | 38 | 0.088 |
| Female | 30 | 19 | | 20 | 29 | |
| T Classification | | | | | | |
| T1~T2 | 39 | 34 | 0.175 | 22 | 51 | 0.470 |
| T3~T4 | 18 | 8 | | 10 | 16 | |
| N Classification | | | | | | |
| ≥1 | 13 | 12 | 0.371 | 7 | 18 | 0.805 |
| <0 | 46 | 28 | | 25 | 49 | |
| Distant metastasis | | | | | | |
| Yes | 2 | 2 | 1 | 3 | 1 | 0.098 |
| No | 57 | 38 | | 29 | 66 | |
| YK-classification | | | | | | |
| 1/2/3 | 41 | 37 | 0.006 | 18 | 60 | 0.0003 |
| 4C/4D | 18 | 3 | | 14 | 7 | |
| Differentiation | | | | | | |
| Well/moderate | 56 | 36 | 0.436 | 30 | 62 | 1 |
| Poor | 3 | 4 | | 2 | 5 | |

## Results

*Immunohistochemical staining for EZH2 protein and BMI1 protein in OSCC and epithelial dysplasia*. BMI1 and EZH2 staining was exclusively nuclear in epithelial cells. Examination of non-neoplastic squamous epithelium revealed no BMI1 or EZH2 immunostaining in normal epithelial cells (Figures 1 and 2). However, a few non-neoplastic epithelial cells stained positive for BMI1 or EZH2 expression. Examination of OSCC tissues revealed BMI1 and EZH2 staining in the majority of cancer cells. The percentage of nuclei staining positively for either EZH2 or BMI1 was significantly higher in OSCC tissue than in normal epithelium and epithelial dysplasia (Figures 1 and 2).

*Aberrant expression of EZH2 and BMI1 protein in resected tissue by immunoblot analysis*. BMI1 and EZH2 protein were detected in OSCC tissue, but not in normal tissue (Figure 3).

*Aberrant expression of EZH2 and BMI1 in OSCC tissue and its association with clinicopathological parameters and outcome*. Among the 99 OSCC cases, aberrant expression of EZH2 protein was observed in 32 (32.3%), and this was significantly associated with mode of invasion. Aberrant expression of BMI1 protein was observed in 59 (59.6%) cases, and this was inversely associated with the mode of invasion (YK-classification) (Table I). There was no association between aberrant BMI1 and EZH2 expression and the survival time of patients with OSCC (Figure 4).

*Association between aberrant EZH2 expression and P53*. To identify biological factors associated with aberrant expression of EZH2 protein, we evaluated expression of P53 protein by immunohistochemistry in the 99 OSCC cases, 32 of which were positive for aberrant EZH2 expression and 67 of which were negative for EZH2 expression. As shown in Table III, aberrant EZH2 expression was significantly associated with P53 protein expression. This association was confirmed in all 99 cases ($p<0.01$).

*Association between transcriptional activity of P53 and EZH2 expression in OSCC cell lines*. SAS/neo cells express wild type *P53* and SAS/mp53 cells express a mutant p53 protein (41). We observed significant up-regulation of *EZH2* mRNA and protein in SAS/mp53 cells relative to SAS/neo cells (Figure 5).

## Discussion

In this study, we conducted immunostaining for EZH2 and BMI1 protein in both epithelial dysplasia and OSCC lesions. In normal oral squamous epithelium, we found no staining

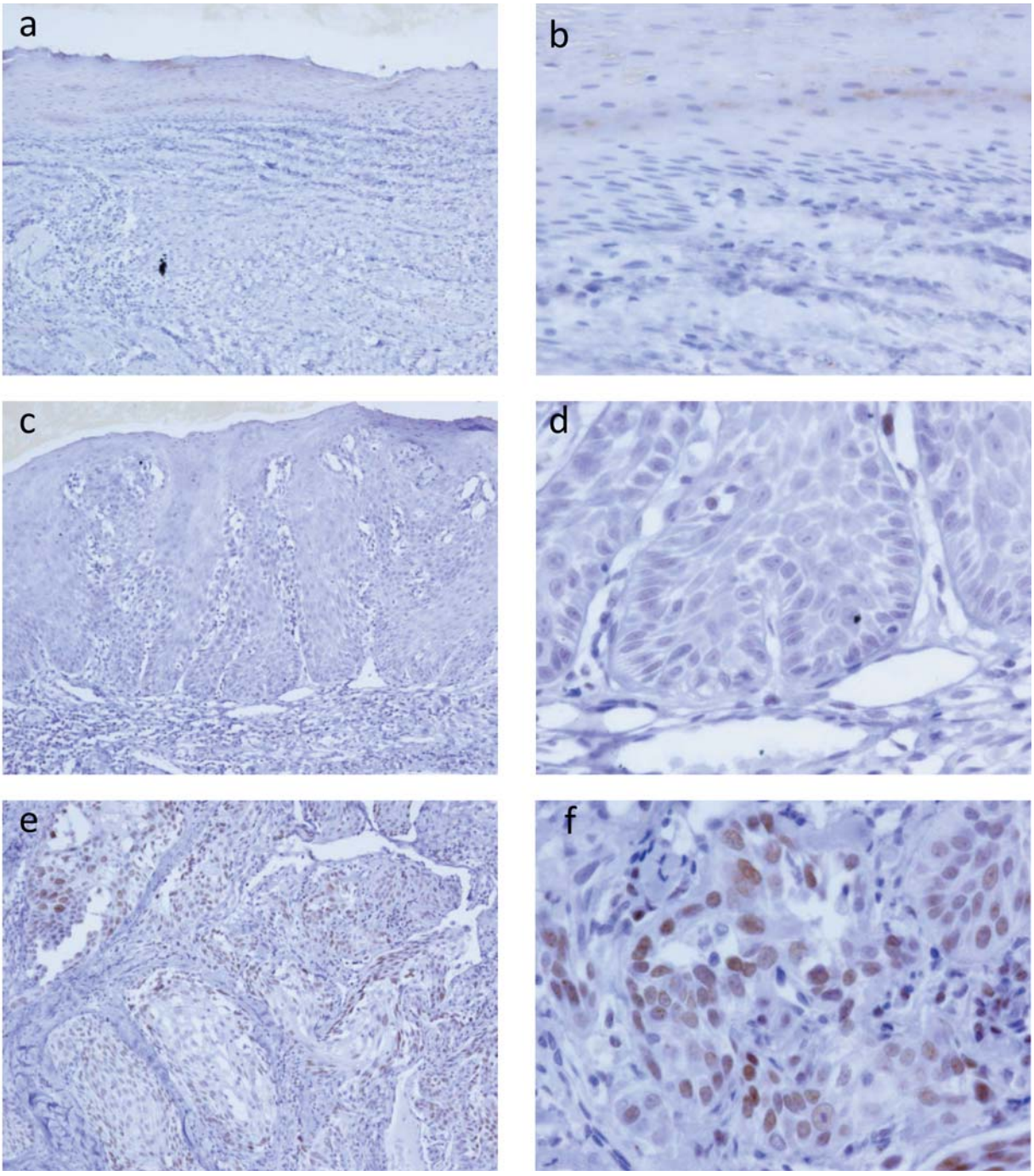


Figure 1. *Immunohistochemistry of B lymphoma Mo-MLV insertion region 1 homolog (BMI1) protein expression. a and b: Normal oral epithelial tissue (×100 and ×400 magnification, respectively). c and d: Weak staining of BMI1 detected in oral epithelium dysplasia (×100 and ×400 magnification, respectively). e and f: Strong staining of BMI1 detected in OSCC (arrow, ×100 and ×400 magnification, respectively). BMI1 expression is mainly localized to the nuclei of tumor cells.*

for EZH2 or BMI1. Conversely, the majority of the cancer cells stained positively for EZH2 and BMI1. The percentage of nuclei with intense staining for either EZH2 or BMI1 was greater than 5% in some OSCC lesions, and was significantly higher than in the oral epithelial dysplasia lesions (Figure 1 and 2). Aberrant expression of EZH2 and BMI1 was present in 32.3% and 59.6%of OSCC tissue, respectively. The results of this study also demonstrate that aberrant expression of the

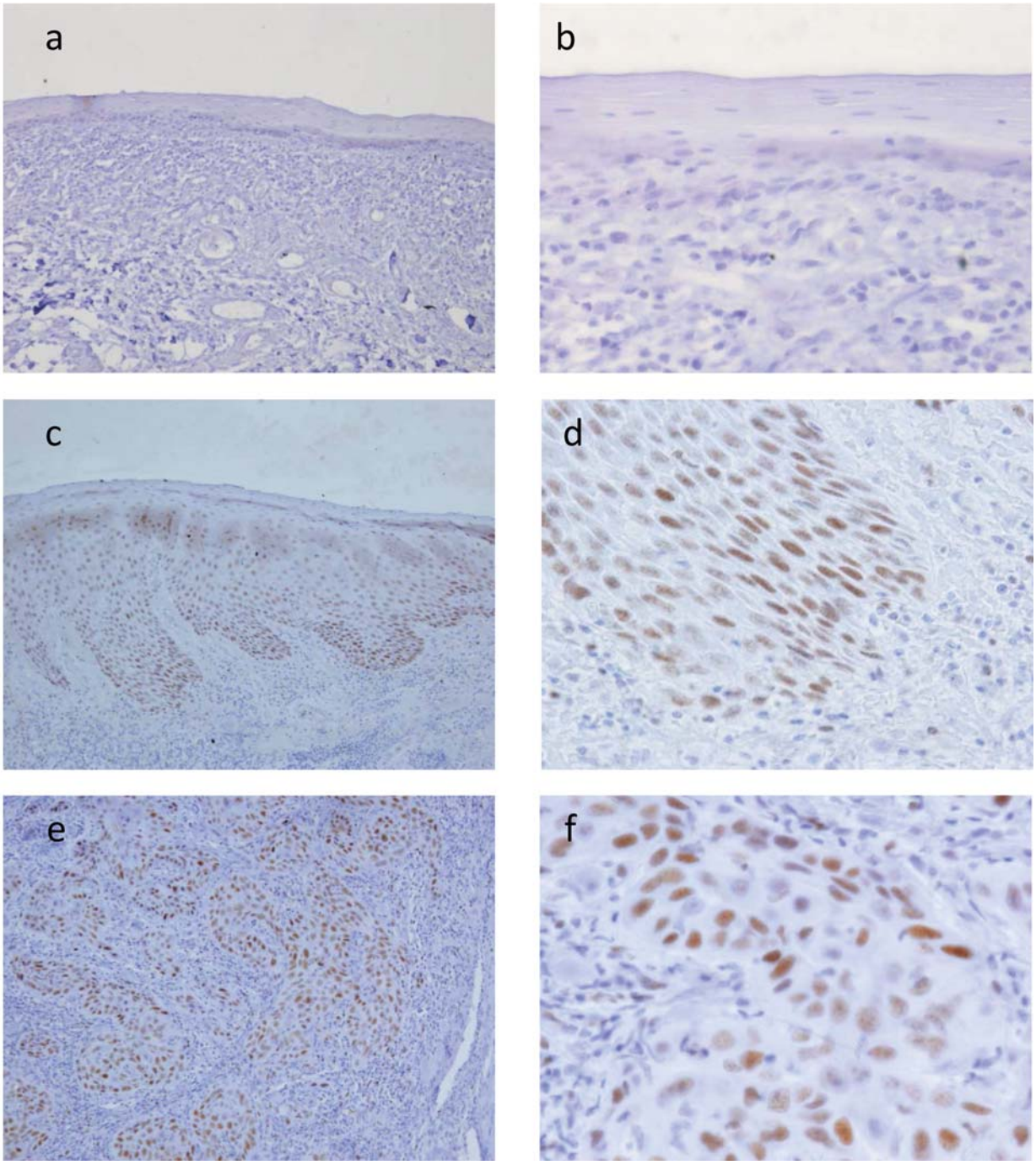


Figure 2. *Immunohistochemistry of Enhancer of zeste homolog 2 (EZH2) protein expression. a and b: Normal oral epithelial tissue (×100 and ×400 magnification, respectively). c and d: Positive staining of EZH2 detected in oral epithelium dysplasia (×100 and ×400 magnification, respectively). e and f: Positive staining detected in OSCC (arrow,×100 and ×400 magnification, respectively).*

EZH2 protein is associated with the mode tumor of invasion. Recent studies have indicated that high EZH2 expression is correlated with poor progression-free survival and poor disease-specific survival in some types of cancer (42). However, our results indicate that aberrant expression of EZH2 and BMI1 was negatively associated with lymph node metastasis and survival. In our study, we also found EZH2 and BMI1 expression in some patients with epithelial

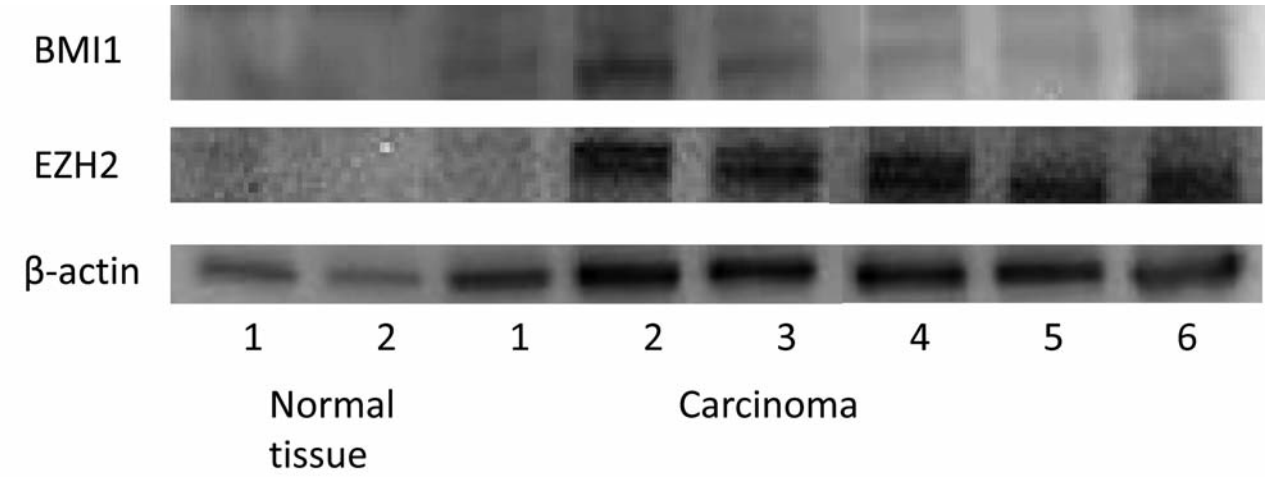


Figure 3. *Representative western blot of B lymphoma Mo-MLV insertion region 1 homolog (BMI1) and Enhancer of zeste homolog 2 (EZH2) in oral tissue. BMI1 and EZH2 protein were detected in oral squamous cell carcinoma (OSCC) but not in normal tissue.*

Table II. *Correlation between the pathogenesis and expression of B lymphoma Mo-MLV insertion region 1 homolog (BMI1) and Enhancer of zeste homolog 2 (EZH2) proteins.*

| | BMI1 expression | | |
|---|---|---|---|
| Pathology | High | Low | |
| Normal (n=12) | 0 | 12 | |
| Dysplasia Mild (n=12) | 2 | 10 | N.S. |
| Moderate (n=11) | 4 | 7 | |
| Severe (n=11) | 3 | 8 | $p<0.05$ |
| Carcinoma (n=99) | 59 | 40 | |
| | EZH2 expression | | |
| | High | Low | |
| Normal (n=12) | 0 | 12 | |
| Dysplasia Mild (n=12) | 1 | 11 | N.S. |
| Moderate (n=11) | 1 | 10 | |
| Severe (n=11) | 2 | 9 | $p<0.05$ |
| Carcinoma (n=99) | 32 | 67 | |

Table III. *Association between aberrant expression of Enhancer of zeste homolog 2 (EZH2) and B lymphoma Mo-MLV insertion region 1 homolog (BMI1) and expression of p53.*

| | Aberrant BMI1 expression | | |
|---|---|---|---|
| | Positive (n=59) | Negative (n=40) | *p*-Value |
| P53 | | | |
| Positive | 33 | 15 | 0.10094 |
| Negative | 26 | 25 | |
| | Aberrant EZH2 expression | | |
| | Positive (n=32) | Negative (n=67) | *p*-Value |
| P53 | | | |
| Positive | 29 | 19 | 0.00049 |
| Negative | 3 | 48 | |

dysplasia. These findings suggest that EZH2 and BMI1 are associated with an early carcinogenesis event.

An interesting finding in this study was that among the several molecules that have been reported to be involved in EZH2 regulation, which are also known to have an important role in OSCC development, aberrant expression of EZH2 was significantly associated only with P53 protein expression in OSCC tissue. Pietersen et al. previously demonstrated a positive association between high EZH2 expression and either P53 mutation or P53 protein expression in breast cancer (37). In agreement with our observations in OSSC tissue samples described above, EZH2 expression in SAS/mp53 cells was significantly higher than in SAS/neo cells. SAS/mp53 cells express a mutant P53 protein that exhibits impaired transcriptional activity, which suggests a reduction in P53 transcription can cause up-regulation of EZH2.

In conclusion, expression of EZH2 and BMI1 was found to be significantly up-regulated in OSCC tissue in comparison with normal epithelium and epithelial dysplasia. Aberrant EZH2 expression in OSCC was found to be significantly associated with the mode of tumor invasion. In addition, aberrant EZH2 expression was found to be significantly

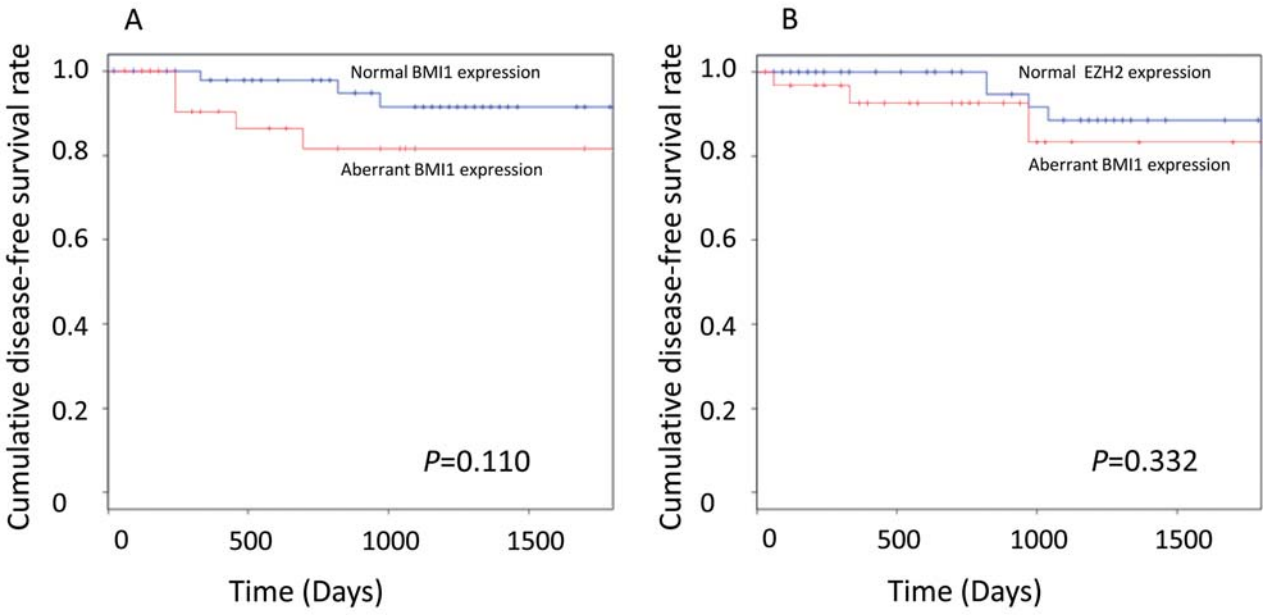


Figure 4. *Disease-free survival curves of patients with OSCC. Kaplan–Meier disease-free survival curves for patients with aberrant expression of B lymphoma Mo-MLV insertion region 1 homolog (BMI1) (a) and Enhancer of zeste homolog 2 (EZH2) (b) reveal no significant differences between the groups.*

associated with P53 alteration. Taken together, our findings suggest that P53 alteration may be involved in dysregulation of EZH2 expression and that aberrant expression of EZH2 may be an early event in the development of OSCC.

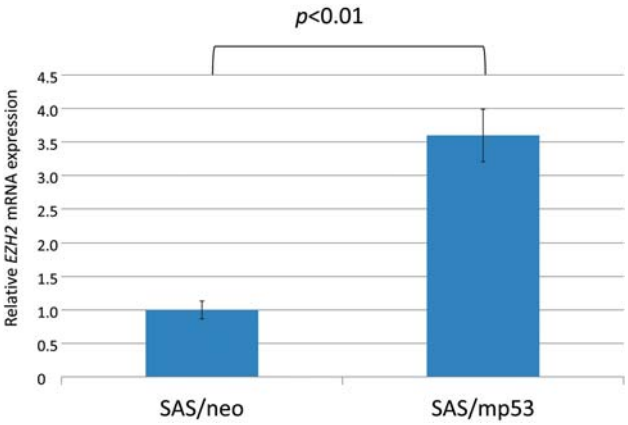


Figure 5. *Expression of Enhancer of zeste homolog 2 (EZH2) mRNA in oral squamous cell carcinoma cell lines. SAS cells transfected with mutant P53 (SAS/mp53) showed high EZH2 expression compared with SAS cells transfected with neo-vector as control (SAS/neo).*

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