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<th>Expression of BMI1 and ZEB1 in epithelial-mesenchymal transition of tongue squamous cell carcinoma</th>
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Expression of BMI1 and ZEB1 in epithelial-mesenchymal transition of tongue squamous cell carcinoma

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Abstract

Introduction: The Epithelial-mesenchymal transition (EMT), the crucial event for the invasion and progression of epithelial carcinogenesis, induces stem-like properties for epithelial cells. Expression of BMI1 which controls self-renewal in stem cells and ZEB1, a transcription factor which regulates EMT were studied on the role in the carcinogenesis process of tongue squamous cell carcinoma (TSCC).

Material and methods: Collagen invasion assay using 2 TSCC cell lines and 64 tongue tissue specimens (32 carcinomas and 32 dysplasias) were used the materials of this study. We assessed protein and mRNA expression levels of BMI1, ZEB1, Vimentin and E-cadherin in both cell lines and tumor tissues.

Results: Both of the protein and mRNA expression of BMI1 and ZEB1 occurred at the invasion of TSCC. The elevated levels of BMI1 and ZEB1 were accompanied by downregulation of E-cadherin and upregulation of Vimentin at the invasive front, induced of EMT both in vivo and in vitro.

Conclusions: We demonstrate that BMI1 and ZEB1 are important factors in associated with promotion of EMT and invasion of TSCC.
Introduction

Head and neck squamous cell carcinoma (HNSCC) including oral cancer is the sixth most prevalent cancer worldwide and accounts for approximately 8-10% of all cancers in Southeast Asia [1, 2]. At present, despite improvements in the diagnosis and management of HNSCC, long-term survival rates have improved only marginally over the past decade [3]. To improve the survival rate of HNSCC patients, investigations the underlying molecular and phenotypic events associated with head and neck squamous tumorigenesis to identify biomarkers for early detection and prognostic stratifications are needed. Recently studies have suggested that the persistent survival of cancer stem cells (CSCs), also known as tumor-initiating cells, may contribute to the aggression and recurrence of HNSCC [4, 5, 6, 7]. These CSCs are key contributors to radioresistance and chemoresistance and are responsible for tumor progression and recurrence after conventional therapy (radiotherapy and chemotherapy) [4, 8].

The epithelial-mesenchymal transition (EMT) is a key developmental program
that CSCs is often activated during cancer development [9, 10]. The occurrence of EMT in cells may lead to the number of changes including loss of cancer cell polarity and downregulation of epithelial cell markers, loss of cell-cell connection, gain of mesenchymal phenotypes along with genetic/epigenetic modifications of various genes. Published reports suggest a direct link between the EMT and the gain of CSCs-like properties [11]. This process is thought to be a critical step in the induction of tumor metastasis and malignancy [12].

BMI1 (B-lymphoma Moloney murine leukemia virus insertion region-1), a member of polycomb group (PcG) genes, is considered to be pivotal in regulating a stemness-related gene maintaining the self-renewal capacity of stem cells through promoting chromatin modifications, and is also known to be deregulated in various human cancers [13, 14, 15, 16]. BMI1 is e a prognostic marker in prostate cancer [17], breast cancer [18], ovarian cancer [19], cervical cancer [20], colorectal cancer [21], lung cancer [22], esophageal cancer [23], gastric cancer [24], and nasopharyngeal cancer [13]. However, the role of BMI1 in maintaining self-renewal properties and tumorigenicity in HNSCC or HNSCC-derived cancer stem cells (CSCs) has yet to be clarified.
ZEB1, a member of the zinc-finger transcription factor family, is one of the master regulators of EMT that mediates invasiveness as well as metastasis in many different types of malignant tumors. ZEB1 induces EMT by suppressing the expression of E-cadherin and contribute to the progression of malignant cancer [25]. ZEB1 is a good predictor of prognosis in breast cancer [26], lung cancer [27], colorectal cancer [28], and esophageal cancer [29]. Extensive studies have revealed that several transcription factors such as ZEB1 work together to regulate the EMT program [30]. However, the role of ZEB1 in HNSCC remains unclear.

Here, we studied several biomarkers, BMI1 and ZEB1, Vimentin, E-cadherin associated with EMT in tongue squamous cell carcinoma (TSCC) cells and tumor specimens to determine their relation to the invasion and progression of TSCC which accounts for approximately 60% of oral squamous cell carcinoma and clarified the significance of BMI1 and ZEB1 in TSCC.
Materials and methods

Carcinoma cell lines and isolation of fibroblasts

Two human tongue squamous cell carcinoma cell lines, TOSCa-2S, TOSCa-23, and human fibroblasts [31]. Human fibroblasts were collected from human oral specimens and used as stromal cells for this assay.

Collagen gel invasion assay and immunohistochemistry of TSCC cells

To conduct the collagen gel invasion assay, we used a 3-dimensional collagen gel culture. Insert chambers with 8 μm pore filter were placed in six 35 mm culture dishes.

First, a collagen solution was poured into insert chambers and incubated at 37℃ for 30 minutes to solidify the gel. Secondly, eight volumes of acid-soluble 0.3% type I collagen solution (Cellmatrix type I-A, pH 3), one volume of 10× concentrated minimum essential medium, and 1 volume of reconstruction buffer (2.2g of sodium bicarbonate and 4.77g of HEPES dissolved in 100mL of 0.05N sodium hydroxide) were mixed. Fibroblasts were added to this solution at a
density of $1 \times 10^5$/ml, then 2ml of this mixture containing fibroblasts was added to the chamber on top of the solid collagen-only layer. After gelation, TSCC cells suspension at a concentration of $1 \times 10^6$ cells per dish was spread on the gel. After incubating for four weeks, both of TSCC cell lines were observed migrating into the underlying gel. The whole collagen gel was fixed with 10% formalin embedded in paraffin, 4µm thinsectioned vertically, stained with hematoxylin-eosin. For immunostaining, antigens were retrieved by heating at 120°C for 20 minutes, cancer cells were identified with Vimentin, E-cadherin, BMI1 and ZEB1 antibodies (Fig 1).

**Analysis of TSCC cell invasion**

The linear borderline formed at the contact points between the cells and the gel was corresponding to a basement membrane-like structure. We judged TSCC cells organized a stratified layer on the gel or contacting with the basement membrane as a preinvasion, and the downgrowth into the gel separating from the basement membrane as an invasion. Four specimens for each cell line were observed, preinvasive cells and invasive cell were counted
and examined the percentage of positive cells and correlation between the group of preinvasive and invasive cells.

**Tissue samples and patients**

Tongue tissue specimens accessed at Oral Pathology of Showa University from 1997 to 2011 were used the materials of this study. A total of 47 patients were eligible of inclusion (24 men and 23 women, median age 58 years, range 30-83 years). All patients had undergone resection of the tongue primary tumor and this study did not include any patient with a distant metastasis and any who had received preoperative therapy.

This study was approved by the Committee on Ethics, Oral Pathology of Showa University, adhered to the principles of the Declaration of Helsinki, and all of the samples were obtained after the patients had provided their informed consent. (Permit number 8, November 2, 2001)

Tongue tissues were surgically resected from patients and the hematoxylin and eosin-stained slides were reviewed. The tissues were immediately sent to Pathology lab, stored at -80°C for immunohistochemistry and real-time RT-PCR.
Sixty-four lesions obtained from fresh-frozen tongue tissue specimens were consisted 32 primary invasive tongue cancer (15 early invasive cancer which do not invade the muscle layer and 17 advanced invasive cancer which invade the muscle layer) and 32 dysplasia (14 mild dysplasia and 18 moderate-severe dysplasia). There were some specimens with invasive cancer and dysplasia simultaneously. The original histological sections and immunostainings were evaluated by a single pathologist without knowledge of clinical data.

**Immunohistochemistry for Tongue tissues**

The frozen tissue slides were cut into 4 \( \mu \)m-thick sections, fixed in 4% paraformaldehyde and treated with 3% hydrogen peroxide in methanol for 10 minutes to block the endogenous peroxidase. Immunostaining was performed with a mouse monoclonal E-cadherin antibody (Santa Cruz Biotechnology, SC-8426, diluted 1:100), or rabbit polyclonal BMI1 antibody (Cosmo Bio Epitomics, T3421, 1:100), or rabbit polyclonal ZEB1 antibody (Santa Cruz Biotechnology, SC-25388, 1:100) overnight at 4\( ^\circ \)C, or mouse monoclonal Vimentin antibody (Dako, #M0725; 1:200) for 30 minutes at room temperature.
After rinsing in phosphate buffered saline, sections were incubated with biotinylated secondary antibody. Detection was performed with diaminobenzidine (DAB) and counterstained with Mayer hematoxylin.

Assessment of immunohistochemical staining

Immunostainings were observed in the parabasal and basal cell layer of the normal squamous epithelium and dysplasia, the outermost layer of cancer nest in the invasive front of the invaded cancer. The semi-quantitative analysis of the stained sections was done by light–microscopy according to the immunoreactive scoring (IRS) system by Remmele and Stegner. Sections were examined at 400×magnification and the staining intensity (SI) was evaluated by comparison with adjacent normal epithelia, which served as a reference for moderate intensity (M). Tumor staining less intense than the basal layer of adjacent normal epithelia was categorized as weak intensity (W), more intense staining was categorized as strong intensity (S), and no staining was categorized as negative (N). We also calculated the percentages of cells with different staining intensities to have ten spots for each slide and the predominant intensity was recorded for
each tumor. Based on the percentage of positive cells (PP), stainings were classified into five grades: grade 0(0%), grade 1 (0-10%), grade 2 (11-50%), grade 3 (51-80%) and grade 4 (80-100%). The product of SI and PP was the IRS. The IRS with points from 0 to 12 was adapted to an additional 3-points IRS classification (Table 1).

Laser microdissection

The 8 μm-thick sliced sample was fixed in 95% ethanol for 5min, and then washed with 70% ethanol, and stained with LCM frozen section staining kit (AMBION). We procured a few hundred cells from cancer tissue and epithelial dysplasia and adjacent normal epithelia in each 15 cases using laser microdissection (PALM MicroBeam, ZEISS) for analysis of gene expression. The mRNA expression were collected from the parabasal and basal cell layer of the normal squamous epithelium and dysplasia, cancer nest in the invasive front of the invaded cancer. The microdissected cells within the cap were covered with 100 μl buffer solution and the capped tube was vortexed. Total mRNA was independently extracted from each population of laser-microdissected cells.
**Quantitative real-time reverse transcription –PCR**

Total mRNA was extracted with RNeasy Plus Micro Kit (QIAGEN) from frozen, microdissected samples of cancer tissue, epithelial dysplasia and adjacent normal epithelia, according to the manufacturer’s instructions. mRNA was reverse transcribed with SuperScript VILO Master Mix (Invitrogen) and cDNA synthesis was performed. Quantitative real-time PCR was performed with an ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems).

The amplification profile was denatured at 95°C for 10 min, followed by 50 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 1 min. The expression levels were quantified using the following Vimentin primer (Hs00185584_m1, Taqman, Applied Biosystems), E-cadherin primer (Hs01023894_m1, Taqman, Applied Biosystems), BMI1 primer (Hs00180411_m1, Taqman, Applied Biosystems), and ZEB1 primer (Hs00232783_m1, Taqman, Applied Biosystems). The geometric mean of the GAPDH (glyceraldehydes-3-phosphate dehydrogenase housekeeping gene) was used as an internal control to normalize the variability in expression levels.

The comparative cycle threshold (CT) method was applied to quantify the
expression levels of mRNAs. The relative amount of each marker was calculated using the equation $2^{-\Delta C_T}$ where $\Delta C_T = (C_{TX} - C_{T GAPDH})$

**Statistical analysis**

Comparison the protein expression levels according to the IRS between two groups was used for the Kruskal Wallis test and the Mann-Whitney *post hoc*, and comparison the mRNA expressions was used for the non-repeated measures ANOVA and *post hoc*. The correlation between the expressions of several biomarkers and TSCC cells was evaluated with the Chi-square test and the Fisher’s exact test, spearman’s correlation. All the statistical analyses were performed using modified EZR (The R Foundation for Statistical Computing, Perugia, Italy) software programs. Two-tailed P-values were calculated, and P<0.05, <0.01 was considered to indicate a statistically significant result.
Results

Protein expression of TSCC cells in invasion assay

In collagen gel invasion assay, we evaluated protein expressions in early invasion carcinogenesis of TSCC cell (TOSCa-2S, TOSCa-23). Only 2.5% of the preinvasive cells, as many as 70.0% of the invasive cells were defined as manifesting high Vimentin expression. We distinguished between fibroblasts and invasive cancer cells in collagen gel stained with Vimentin. 72.3% of the preinvasive cells, only 3.6% of the invasive cells were defined as manifesting high E-cadherin expression. 62.4% of the preinvasive cells, 73.0% of the invasive cells were defined as manifesting high BMI1 expression, and 60.2% of the preinvasive cells, 73.5% of the invasive cells were defined as manifesting high ZEB1 expression. Protein expressions of all markers were significantly different between preinvasive cells and invasive cells (P<0.001, P<0.01; Fig 2).

Protein and mRNA expression of tongue cancer and dysplasia specimens

We compared of immunohistochemical expression among the five groups,
normal squamous epithelium, mild dysplasia, moderate-severe dysplasia, early invasive cancer and advanced invasive cancer. BMI1 immunoexpression was mainly localized to the nuclei of normal squamous epithelium and dysplasia but detected in both the nuclei and cytoplasm of the majority of tongue cancer. BMI1 high immunoexpression (IRS-classification: 12) was observed in 84.4% (27 of 32) of invasive cancer. ZEB1 immunoexpression was detected in cytoplasm of all samples and ZEB1 high immunoexpression was observed in 50.0% (16 of 32) of invasive cancer. (Fig 3)

E-cadherin protein expression level was significantly decreased in moderate-severe dysplasia and invasive cancer compared with adjacent normal squamous epithelia (P=0.0021, P<0.001, P<0.0001; Fig 4). Vimentin and BMI1 protein expression levels were significantly increased in invasive cancer, including early and advanced invasive cancer, (P=0.0074, <0.001, P=0.04, 0.0001; Fig 4), and ZEB1 protein expression levels was significantly increased only in advanced invasive cancer (P=0.014; Fig 4), compared with adjacent normal squamous epithelia.

E-cadherin mRNA expression level was significantly decreased in
moderate-severe dysplasia and invasive cancer compared with adjacent normal squamous epithelia (P=0.039, P=0.019; Fig 5). Vimentin, BMI1 and ZEB1 mRNA expression levels were significantly increased in invasive cancer compared with adjacent normal squamous epithelia (P=0.047, P=0.036, P=0.045; Fig 5).

Elevated levels of BMI1 were accompanied by downregulation of E-cadherin and upregulation of Vimentin at the invasive front, demonstrated a significant negative correlation between BMI1 and E-cadherin protein and mRNA expressions (P= 0.0097; Fig 6A, P= 0.018; Fig 6B), and a significant positive correlation between BMI1 and Vimentin protein and mRNA expressions. (P=0.035; Fig 6A, P=0.0008; Fig 6B). There was a significant positive correlation between ZEB1 and Vimentin mRNA expressions, between BMI1 and ZEB1 mRNA expressions (P<0.001, P=0.024; Fig 6B).

**Discussion**

EMTs are encountered in three distinct biological settings [32]. Type 1 of EMTs are associated with implantation, embryonic gastrulation, gives rise to the
mesoderm and endoderm and to mobile neural crest cells and organ development are organized. Type 2 of EMTs are re-engaged in the context of inflammation and fibrosis. These EMTs continued to occur until infections are removed over extended periods of time and the tissue is repaired. Type 3 of EMTs occur in the context of tumor growth and cancer progression, and the epithelia can transform into cancer cells that later undergo EMTs that enable invasion and metastasis. All EMTs have in common and E-cadherin transcriptional repression is characteristic of EMT. Given that the downregulation of E-cadherin is tightly associated with EMT program and the invasion of cancer, it is worth remembering that E-cadherin loss has long been associated with the progression of papilloma to invasive carcinoma [33].

In the present study, we examined the possible involvement in tongue carcinogenesis by comparison of BMI1 and ZEB1, E-cadherin, Vimentin protein and mRNA expression levels in normal, dysplasia and TSCC tissues and TSCC cells.

In TSCC cells invasion assay, we demonstrated that downregulation E-cadherin protein expression observed in 96.4% of the invasive TSCC cells were
demonstrated the promotion of EMT program and TSCC cells invasion. We revealed that BMI1 and ZEB1 protein overexpression were observed in 73% of the invasive TSCC cells compared with 60% of the preinvasive TSCC cells. This finding indicated that BMI1 and ZEB1 protein overexpression are associated with the EMT program and the TSCC cells invasion.

In TSCC tissues, BMI1 protein and mRNA expression levels were significantly increased in invasive tongue cancer, including early and advanced invasive cancer, compared with adjacent normal squamous epithelia. At the invasive front, elevated levels of BMI1 were accompanied by downregulation of E-cadherin and upregulation of Vimentin, demonstrated a significant negative correlation between BMI1 and E-cadherin expressions, a significant positive correlation between BMI1 and Vimentin expressions. These data suggest that BMI1 overexpression at both mRNA and protein levels was involved in the invasion and progression of TSCC. These findings are in agreement with previous studies of other epithelial malignancies and further support an important role for BMI1 activation in the downregulation of E-cadherin and the induction of EMT.

For instance, Song et al. demonstrated that Bmi-1 mRNA and protein
expressions were found to correlate with the invasion of nasopharyngeal carcinoma [13]. Yang et al. showed that BMI1 is essential for EMT during tumor development in head and neck cancer patients [14]. MK Kang et al. showed Bmi-1 overexpression was observed in 100% of the preneoplastic oral mucosal tissue which included those with mild, moderate or severe epithelial dysplasia [34]. This divergence from our results may be due to the different pathophysiology of oral squamous cell carcinoma such as non-keratinising type or keratinising of carcinoma, but our material is a keratinizing SCC of tongue carcinoma. The former study constantly presented in a small series of patients (N=8, 10) with oral dysplastic and carcinoma tissue, but our research data consist of 64 cases. Interestingly, V Hayry et al. showed a significant negative correlation between Bmi-1 protein expression and the recurrence of tongue cancer [35]. This divergence from our results may be due to selecting part of tissue which detached with 1mm punch from the surface epithelium and a central of the tumor and the invading front. Balasubramanian et al. reported the expression of BMI1 in basal and suprabasal keratinocytes and is not present in surface epithelium [36]. Recently study showed that in the invading front, BMI is
highly enriched in CSCs but there is not Bmi-1 expression in all cancer cells [37].

In the present study, we just evaluate BMI1 protein expression and mRNA expression of cancer nest cells in the invasive front.

The overexpression of ZEB1 was observed in colorectal cancer and esophageal cancer [28, 29], suggesting an important role in tumorigenesis. EMT-induced ZEB1 was previously reported to be associated with cancer progression [38]. In the present study, we confirmed that ZEB1 protein and mRNA expression levels were significantly increased in advanced invasive cancer compared with adjacent normal squamous epithelia, consistent with the results of previous studies and suggested to be associated with cancer progression.

On the other hand, ZEB1 was found to be responsible for downregulation of basal membrane constituents at the invasive front of colorectal carcinoma [29] and Takehiko Y et al. showed that in esophageal SCC, ZEB1 targeting by miR-150 could suppress E-cadherin repression, Vimentin expression, migration ability, and tumorigenicity [30]. In this study, we indicated that there was a significant positive correlation between ZEB1 and Vimentin mRNA expressions, but was a no significant correlation between ZEB1 and E-cadherin expressions.
A hallmark for EMT is the loss of E-cadherin expression, however, ZEB1 with the EMT process of TSCC may not suppress E-cadherin expression.

In this study, we demonstrated that elevated level of BMI1 was accompanied by downregulation of E-cadherin and upregulation of Vimentin, and elevated level of ZEB1 was accompanied upregulation of Vimentin at the invasive front of TSCC.

The recent study found that the EMT has important roles in cancer invasion and metastasis and cancer stem cell properties [4, 9]. The study demonstrated that ZEB1 and ZEB2 are key modulators of CSC properties in HNC, including EMT, metastasis, and drug resistance [39]. At this time, this study indicated that activation of BMI1, stem cell-like marker, is associated with promotion of EMT and invasion in TSCC and further investigation into the role and mechanisms of BMI1 and ZEB1 in TSCC is require.

In conclusion, BMI1 and ZEB1 are important factors in associated with promotion of EMT and invasion of TSCC.
Acknowledgements

We appreciate the technical help from the Showa University Pathology Department.

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<table>
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<th>Percentage of positive cells</th>
<th>$X$</th>
<th>intensity of staining</th>
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<td>0 = no positive cell</td>
<td>0</td>
<td>no color reaction</td>
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<tr>
<td>5 - 8</td>
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<td>9 - 12</td>
<td>2 more than normal positive</td>
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(Remmele and Stegner 1987)

Table 1 Immunoreactive score (IRS) and IRS classification scoring systems
Fig 1  Immunoexpression of TSCC cells in collagen gel invasion assay
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<tr>
<th></th>
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<th>ZEB1 expression</th>
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<td>p-value</td>
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<td>preinvasive cells</td>
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<td>79 (70.0%)</td>
<td>34 (30.0%)</td>
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<td>preinvasive cells</td>
<td>334 (72.3%)</td>
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Fig 2 Protein expression of TSCC cells in collagen gel invasion assay
Fig 3 Immunoexpression of tongue tissues

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<th>Severe dysplasia</th>
<th>Advanced invasive carcinoma</th>
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<td>H&amp;E</td>
<td>Vimentin</td>
<td>E-cadherin</td>
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×400
Fig 4 Protein expressions of tongue tissues according to the IRS

* Dys: dysplasia, Ca: invasive carcinoma
Fig 5 mRNA expressions of tongue tissues

* Dys: dysplasia, Ca: invasive carcinoma
Fig 6 Correlation of the IHC grading of protein expressions (A) and Correlation of mRNA expressions (B)

### Table A: Bmi-1 expression and P-value

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<th>Bmi-1 expression</th>
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### Table B: Vimentin expression

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<th>Bmi-1 expression</th>
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