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Polycomb group protein EZH2-mediated E-cadherin repression promotes metastasis of oral tongue squamous cell carcinoma

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Abstract

Enhancer of zeste homolog 2 (EZH2) is a critical component of the polycomb-repressive complex 2 (PRC2) that regulates many essential biological processes, including embryogenesis and many developmental events. The oncogenic role of EZH2 has recently been implicated in several cancer types. In this study, we first confirmed that the over-expression of EZH2 is a frequent event in oral tongue squamous cell carcinoma (OTSCC). We further demonstrated that EZH2 over-expression is correlated with advanced stages of the disease and is associated with lymph node metastasis. Statistical analysis revealed that EZH2 over-expression was correlated with reduced overall survival. Furthermore, over-expression of EZH2 was correlated with reduced expression of tumor suppressor gene E-cadherin. These observations were confirmed *in vitro*, in which knockdown of EZH2 induced E-cadherin expression and reduced cell migration and invasion. In contrast, ectopic transfection of EZH2 led to reduced E-cadherin expression and enhanced cell migration and invasion. Furthermore, EZH2 may act on cell migration in part by suppressing the E-cadherin expression. Taken together, these data suggest that EZH2 plays major roles in the progression of OTSCC, and may serve as a biomarker or therapeutic target for patients at risk of metastasis.

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Keywords

EZH2; E-cadherin; metastasis; prognosis; squamous cell carcinoma

Introduction

Oral tongue squamous cell carcinoma (OTSCC) is one of the most common cancers within the oral cavity. According to the American Cancer Society, an estimated 10,990 new cases of tongue cancer are expected each year, accounting for approximately 30% of all oral cavity and pharynx cancers [1]. OTSCC is significantly more aggressive than other forms of oral cancer, with a propensity for rapid local invasion and spread, and a high recurrence rate [2–4]. The major causes of OTSCC-related deaths are local/regional relapse and metastasis. It has been reported that 40% of all OTSCC patients have neck metastasis at the time of diagnosis and 20–40% of patients with early-stage OTSCC (T1/T2N0) showed occult nodal metastasis [5–8]. Improvement in patient survival requires a better understanding of tumor metastasis so that aggressive tumors can be detected early in the disease process and targeted therapeutic interventions can be developed.

Polycomb group (PcG) proteins are epigenetic regulators that function through the formation of polycomb repressor complexes (PRC), including PRC1 and PRC2, which modify chromatin and repress gene expression. Enhancer of Zeste Homologue 2 (EZH2), the catalytic subunit of PRC2, has a histone methyltransferase activity for the trimethylation of histone 3 on lysine 27 (H3K27me3). Generation of the H3K27me3 mark by PRC2 establishes a strong repressive signal for gene expression. The over-expression of EZH2 has been consistently observed in a number of different cancer types [9–13], including oral cancer [14]. However, the effect of EZH2 on metastasis and progression of OTSCC has not been fully defined. In the present study, we assessed the clinical/prognostic significance of EZH2 over-expression in an OTSCC cohort. In addition, the underlying mechanisms involving the pro-metastatic role of EZH2 were investigated.

Materials and Methods

Patient

Archived tissue samples from 67 cases of OTSCC patients who were diagnosed and underwent surgeries resection between 1996 and 2005 at the Department of Oral and Maxillofacial Surgery, Hospital of Stomatology, Sun Yat-sen University were utilized in this study. Clinical characterization of the TSSC patients is summarized in Supplementary Table 1. All patients received surgery with curative intent (resection of the primary tumor and radical neck dissection) between May 1996 and June 2005. None of the patients received any form of adjuvant therapy prior to their surgery. The tumor extent was classified according to the TNM system by UICC, and the tumor grade was classified according to the WHO classification of histological differentiation. Survival was calculated based on the date of surgery and the date of the last follow-up (or death). Median duration of follow-up was 65 months (range 3–120). This study was approved by the ethical committee of Sun Yat-sen University.

Immunohistochemical analysis

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded tissue sections. Representative sections were first stained with H&E and histologically evaluated by a pathologist. The selected sections were deparaffinized, quenched for endogenous peroxidase activity, and rehydrated as described previously [15]. Sections were then blocked with 10% normal serum for 15 min at 37 °C followed by incubation with rabbit anti-EZH2

(Cell signaling, USA), or mouse anti-E-cadherin (BD Bioscience, USA) at a dilution of 1:100 for 16 h at 4 °C. After washing three times in PBS, the sections were incubated with secondary antibody conjugated to biotin for 10 min at room temperature. After additional washing in PBS, the sections were incubated with streptavidin-conjugated horseradish peroxidase enzyme for 10 min at room temperature. Following final washes in PBS, antigen-antibody complexes were detected by incubation with a horseradish peroxidase substrate solution containing 3, 3'-diaminobenzidine tetrahydrochloride chromogen reagent, and counterstained with hematoxylin. Slides were rinsed in distilled water, cover-slipped using aqueous mounting medium, and allowed to dry at room temperature.

The relative intensities of the completed immunohistochemical reactions were evaluated using light microscopy by 3 independent trained observers who were unaware of the clinical data. All areas of tumor cells within each section were analyzed. The adjacent non-cancerous histological normal epitheliums were used as control. All tumor cells in ten random high power fields were counted. Immunoreactivity was semi-quantitatively evaluated on the basis of staining intensity and distribution using the immunoreactive score, where immunoreactive score (IS) = intensity score × proportion score. The intensity score was defined as 0: negative; 1: weak; 2: moderate; or 3: strong, and the proportion score was defined as 0: negative; 1: 10%; 2: 11–50% 3: 51–80% or 4: > 80% positive cells. The immunoreactive score ranged from 0 to 12. The immunoreactivity was divided into two groups on the basis of the immunoreactive score: low immunoreactivity was defined as a total score of 0–4, and high immunoreactivity was defined as a total score >4.

Cell Culture and the treatments of cell lines

Human OTSCC cell lines (SCC9 and UM1) were maintained in DMEM/F12 medium supplemented with 10% FBS, 100 U/mL penicillin and 100 µ/mL streptomycin (GIBCO, USA). For functional analysis, gene specific siRNA against EZH2 and E-cadherin (On-TargetPlus SMARTpool, Dharmacon, USA) or control siRNA was transfected into cells using DharmaFECT Transfection Reagent 1 as described previously [16,17]. The EZH2 expression vector (pCMV6-EZH2, OriGene Technologies, USA) or empty vector was transfected into cells using Lipofectamine 2000 (Invitrogen, USA).

Fluorescent immunocytochemical analysis

Cells were fixed on the slides and permeabilized as previously described [18], and then blocked with 5% BSA for 30 minutes, followed by incubation with primary antibodies (E-cadherin, BD Bioscience, USA and EZH2, Cell signaling, USA) at 4°C for 16 h. The slides were then washed with PBS and incubated with Rhodamine-conjugated secondary antibody (for E-cadherin) or FITC-conjugated secondary antibody (for EZH2) for 1 hour at 37°C. The slides were then washed with PBS and incubated with 53g/ml DAPI for nuclear staining. The cells were visualized for immunofluorescence with a laser scanning Zeiss microscope system (Zeiss Axivert 400C, Germany).

Western blot analysis

Western blots were performed as described previously [17] using antibodies specific to EZH2, SUZ12, H3K27me3 (Cell Signaling, USA), E-cadherin (BD Biosciences, USA), EED (Millipore, USA), beta-actin (Sigma-Aldrich, USA), and a Immuno-Star HRP Substrate Kit (BIO-RAD, USA).

In vitro cell migration and invasion assay

The *in vitro* cell migration and invasion were measured using the BD BioCoat system (BD Biosciences, USA) following the manufacturer's instructions. In brief, cells were seeded in

the upper chambers, and culture medium with 10% FBS was added to the lower chambers. For invasion assay, transwell inserts coated with Matrigel were used. After 24 h incubation, cells that migrated to the reverse side of inserts were stained with Diff-Quik stain kit (Polyscience, USA) and quantified.

Statistical analysis

Data were analyzed using the Statistical Package for the Social Science (SPSS, Chicago, USA), Version 17.0. Spearman Correlation Coefficient was used to assess correlations among the gene expression and clinical and histopathological parameters. One-way ANOVA was used to assess the association of EZH2 expression with pN. Kaplan-Meier plots were constructed to present survival outcomes. Cox regression was used for both univariate and multivariate analysis. For multivariate analysis, grade, tumor size, pathological T-stage (pT), pathological N-stage (pN), clinical stages, and the expression of EZH2 and E-cadherin were considered as co-variants. For all statistical analyses, $p < 0.05$ was considered statistically significant.

Results

The expression of EZH2 in OTSCC

The expression of the EZH2 gene was examined by immunohistochemistry (IHC) in an OTSCC patient cohort ($n = 67$) (Supplementary Table 1). As illustrated in Figure 1A, in normal epithelium ($n = 10$), EZH2 was detectable only in basal layers. In cancer cells, predominant nuclei staining of EZH2 was observed. Semi-quantitative analysis revealed that EZH2 is significantly over-expressed in OTSCCs (immunoreactive score = 4.1 ± 2.9) when compared to normal mucosa tissue (immunoreactive score = 0.6 ± 0.7 , $p = 0.0003$). Among 67 cases of OTSCC samples that we examined, 44 cases (65.7%) exhibited low nuclear staining for EZH2 (Figure 1B) and 23 cases (34.3%) exhibited high staining for EZH2 (Figure 1C).

Because over-expression of EZH2 has been linked to the reduced expression of tumor suppressor gene E-cadherin [19], the expression of E-cadherin in these cases was also examined by IHC. As shown in Figure 1D, strong staining of E-cadherin was detected at the cytoplasmic membrane and the intercellular borders in the normal epithelium. Among 67 cases of OTSCC that we examined, high E-cadherin expression was observed in 27 cases (40.3%) (Figure 1E), and reduced E-cadherin expression was found in 40 cases (59.7%) (Figure 1F).

Correlation among EZH2 and E-cadherin expression, and clinicopathological features in OTSCC

Correlations were tested among gene expression (e.g., EZH2 and E-cadherin), clinical and pathological features for both OTSCC patient cohorts (Table 1). The EZH2 expression was correlated with tumor size ($P < 0.01$), grade ($P < 0.05$), pT stage ($P < 0.01$), pN ($P < 0.01$) and clinical stage ($P < 0.01$). The E-cadherin expression was inversely correlated with pT stage ($P < 0.01$). Furthermore, the expression of EZH2 was inversely correlated with E-cadherin expression ($P < 0.05$) (Table 1, Supplementary Figure 1A, and Supplementary Figure 2). As shown in Table 2, the expression of EZH2 was significantly up-regulated in OTSCC cases with lymph node metastasis (pN+) as compared to those with pathologically negative nodes (pN-). In contrast, the E-cadherin expression was significantly down-regulated in OTSCC cases with pathologically positive nodes (pN+) as compared to those with pathologically negative nodes (pN-).

The prognostic value of EZH2 deregulation for OTSCC patients

As illustrated in Figure 2A, a statistically significant difference in prognosis was observed between the high EZH2 expression group (5-year survival rate < 20%) and the low EZH2 expression group (5-year survival rate > 70%). The differences in survival were also observed when patients were grouped based on the expression of E-cadherin (Figure 2B). A striking difference in overall survival was observed when the cases with both high EZH2 expression and low E-cadherin expression were compared with cases with both low EZH2 expression and high E-cadherin expression (5-year survival rate < 10%, and > 90%, respectively) (Figure 2C). As illustrated in Table 3, univariate analysis indicated that tumor size, grade, pathological T-stage, lymph node metastasis, clinical stage, EZH2, and E-cadherin were all significant prognostic factors for patients with OTSCC. Multivariate analysis showed that tumor size, grade, pathological T-stage, lymph node metastasis, clinical stage, EZH2, and E-cadherin were all independent prognostic factors for OTSCC patients.

The effects of EZH2 on cell migration and invasion in OTSCC cell lines

UM1 is an OTSCC cell line that expresses high levels of EZH2 and exhibits enhanced cell migration and cell invasion, and SCC9 is an OTSCC cell line that expresses low levels of EZH2 and exhibits minimum cell migration and cell invasion. When UM1 cells were treated with EZH2-specific siRNA, the level of H3K27me3 was reduced and the E-cadherin expression was enhanced (Figure 3A). The EZH2 knockdown-induced upregulation of E-cadherin expression was also confirmed at mRNA level and promoter activity level (Supplementary Figure 1B and 1C). In contrast, ectopic transfection of EZH2 to SCC9 cells led to increased level of H3K27me3 and reduced E-cadherin. This is in agreement with the previous finding that EZH2 specifically suppresses E-cadherin expression by introducing H3K27me3 to the promoter of E-cadherin gene [19]. We also examined the expression of Eed and SUZ12, two additional core components of the PRC2 complex. While no change in Eed expression was observed, a slight decrease in Suz12 was observed in UM1 cells treated with EZH2 siRNA, and an apparent increase in Suz12 was observed in SCC9 cells transfected with EZH2. As shown in Figure 3B and 3C, knock-down of EZH2 in UM1 cells led to reduced cell migration and invasion. In contrast, ectopic transfection of EZH2 to SCC9 cells resulted in enhanced cell migration and invasion. The effect of EZH2 on E-cadherin expression was also confirmed by immunofluorescence analysis (Figure 3D and 3K).

As illustrated in Figure 4, when UM1 cells were treated with EZH2 specific siRNA, the cell migration and invasion were reduced significantly. No apparent change was observed when cells were treated with E-cadherin specific siRNA. However, when UM1 cells were co-treated with both EZH2 siRNA and E-cadherin siRNA, the EZH2 knockdown-induced reduction in cell migration was partially reversed. No statistically significant change in cell invasion was observed in cells treated with EZH2 siRNA when compared to cells with knock-down of both EZH2 and E-cadherin. These results indicate that down-regulation of E-cadherin is at least partially involved in the EZH2-mediated regulation of cell migration.

Discussion

Polycomb group (PcG) proteins are a family of epigenetic modifiers that direct cellular fates during embryogenesis by controlling the expression of homeotic genes and other developmental regulators [20]. The oncogenic role of EZH2 has recently been suggested in several cancer types [9–13], including oral cancer [14]. However, the deregulation of EZH2 in OTSCC and its role on metastasis and progression of OTSCC has not been investigated. In the present study, we examined the expression of EZH2 in an OTSCC cohort, and demonstrated that over-expression of EZH2 is a frequent event in OTSCC. This finding

provides evidence that deregulation of EZH2 may play an important role in the tumorigenic process of OTSCC. Further analyses demonstrated that over-expression of EZH2 was positively correlated with advanced disease stages, and was associated with lymph node metastasis. Furthermore, we found that over-expression of EZH2 in OTSCC was a strong and independent predictor of short overall survival. Thus, EZH2 expression appears to have the potential to predict the prognosis of OTSCC patients. The examination of EZH2 expression by IHC, therefore, could be used as an effective tool in identifying those OTSCC patients at increased risk of tumor progression and metastasis. These findings underscore a critical role of EZH2 in the tumorigenesis of OTSCC.

To date, however, the molecular mechanisms by which EZH2 regulates cancer cell migration/invasion remain unclear. A recent study reported that over-expression of EZH2 in cancer cells down-regulates the expression of E-cadherin gene through histone H3K27 trimethylation at the E-cadherin gene promoter [19]. E-cadherin is a tumor-suppressor gene that inhibits migration and invasion of the epithelial tumor cells [21], suppresses epithelial-mesenchymal transition (EMT) [22], and may play a role in the Wnt signaling pathway [23]. Knock-down of EZH2 *in vitro* has been shown to restore E-cadherin expression [9,24]. In the present study, we demonstrated that over-expression of EZH2 is a frequent event in OTSCC, and an inverse correlation of EZH2 expression and E-cadherin expression was observed in our OTSCC patient cohort. Our *in vitro* studies confirmed that knock-down of EZH2 restored E-cadherin expression in OTSCC, and we also demonstrated that knock-in of EZH2 suppressed the E-cadherin expression. The EZH2 knock-down-induced E-cadherin expression is accompanied by reduced cell migration and invasion, and the EZH2 knock-in-mediated up-regulation of E-cadherin is accompanied by enhanced cell migration and invasion. Furthermore, our results demonstrated that EZH2 knock-down mediated inhibition of cell migration was partially reversed by simultaneous knock-down of E-cadherin. This reversal suggested that the effect of EZH2 on cell migration is achieved, at least in part, through regulating E-cadherin expression. However, direct knockdown of E-cadherin by siRNA has a minimal effect on cell migration and invasion in the cell line we tested. This observation indicated that while down-regulation of E-cadherin is involved in EZH2-mediated cell migration and invasion, other EZH2-regulated factors are also involved in this event. It is worth knowing that, deregulation of EZH2 has also been linked to a number of signaling pathways that are relevant to cancer progression and metastasis, including Wnt signaling, Ras and NF-kappa B signaling, and BMP and Notch signaling pathways [25]. A recent study also demonstrated that overexpression of EZH2 is associated positively with overexpression of cyclin D1 in head and neck cancer [26]. It is possible that in addition to the direct repression of E-cadherin gene, EZH2 may regulate E-cadherin by indirect mechanism(s) that involve these EZH2-regulated factors and signaling pathways. Additional studies will be needed to examine the functional relevance of these signaling pathways in E-cadherin repression and lymph node metastasis in OTSCC.

In summary, we described the expression pattern of EZH2 in OTSCC. Our results provide evidence suggesting a critical role of EZH2 in the acquisition of an aggressive and/or metastatic phenotype in OTSCC. Furthermore, EZH2-mediated cell migration is achieved, at least in part, by down-regulating E-cadherin expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

EMT	epithelial-mesenchymal transition
EZH2	enhancer of zeste homolog 2
H3K27me3	trimethylation of histone 3 on lysine 27
IS	immunoreactive score
OTSCC	oral tongue squamous cell carcinoma
pN	pathological N-stage
pT	pathological T-stage
PcG	polycomb group
PRC2	polycomb-repressive complex 2

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Figure 1. Immunohistochemistry analyses of EZH2 and E-cadherin expression in OTSCC
Immunohistopathological analyses were performed as described in Material and Methods to examine the EZH2 and E-cadherin respectively on **A** and **D**: adjacent normal epithelium (n = 10); **B** and **E**: well differentiated primary (n = 48); **C** and **F**: moderate or poor differentiated OTSCC (n = 19). Representative images of $\times 200$ magnification were presented. Scale bar = 200 μm .

Figure 2. The effects of EZH2 and E-cadherin on the prognosis OTSCC patients

Kaplan-Meier plots of overall survival in patient groups defined by the expression levels of EZH2 (A) and E-cadherin (B). In addition, the overall survival between EZH2 high/E-cadherin low subgroup and EZH2 low/E-cadherin high subgroup was also compared (C). The differences in survival rates are statistically significant ($p = 8.06 \times 10^{-7}$, $p = 5.81 \times 10^{-5}$ and $p = 1.35 \times 10^{-9}$, respectively, log-rank test).

Figure 3. EZH2 regulates E-cadherin expression, cell migration and invasion

UM1 cells were transiently transfected with EZH2 specific siRNA or control siRNA. SCC9 cells were transiently transfected with an EZH2 expression vector, or an empty vector. The expression of EZH2, Suz12, Eed, E-cadherin and the level of H3K27me3 were measured by Western blots (A). Cell migration (B) and invasion (C) were measured by trans-well assays. Fluorescent immunocytochemistry analyses were performed to confirm the expressional changes of E-cadherin (red, D–G) and EZH2 (green, H–K) in the intact cells. Data represents at least 3 independent experiments with similar results. * indicates $p < 0.05$.

Figure 4. Down-regulation of E-cadherin is involved in the EZH2-mediated cell migration
 UM1 cells that were treated with siRNA specific to EZH2 or control siRNA, were co-treated with or without siRNA specific to E-cadherin. The expression of E-cadherin and EZH2 (A), the cell migration (B) and invasion (C) were measured. * indicates $p < 0.05$.

Table 1

Correlations among clinical and histopathological features of primary OTSCC [†]

	sex	age	tumor size	grade	pT	pN	C stage	E-cad	EZH2
sex		-0.19	0.14	0.03	0.12	0.00	0.08	0.08	0.11
age			-0.07	-0.05	-0.02	0.06	0.00	0.17	-0.12
tumor size				0.15	0.92**	0.51**	0.84**	-0.13	0.36**
grade					0.19	0.26*	0.17	-0.08	0.31*
pT						0.60**	0.94**	-0.18	0.43**
pN							0.76**	-0.41**	0.32**
C stage								-0.21	0.40**
E-cad									-0.30*
EZH2									

[†] Spearman correlation coefficients were presented.
The expression levels of E-cadherin and EZH2 were generated from semi-quantitative immunohistochemical analysis.
pT: pathological T-stage; pN: pathological N-stage; C stage: clinical stage.
* p < 0.05.
** p < 0.01. The p values were computed using Fisher's transformed z-score test.

Table 2

Association of EZH2 and E-cad expression and lymph node metastasis in OTSCC[‡]

	n	E-cad		EZH2	
		average	variance	average	variance
pN+	27	1.74	0.81	5.15	2.86
pN-	40	2.4	0.84	3.40	2.66
		p = 0.002		p = 0.013	

[‡]One-way ANOVA was used to assess the association of EZH2 and E-cad expression with the pN status.
The expression levels of EZH2 and E-cad were generated from semi-quantitative immunohistochemistry analysis.

Table 3The effects of clinical and histopathological parameters on prognosis of OTSCC patients[†]

	Univariate Analysis		Multivariate Analysis	
	HR (95% CI)	p value	HR (95% CI)	p value
Sex	0.918 (0.445, 1.894)	0.81700		
Age	1.010 (0.990, 1.037)	0.44133		
Tumor size	1.767 (1.401, 2.230)	<0.00001	2.034 (1.549, 2.672)	<0.00001
Grade	2.380 (1.383, 4.095)	0.00173	2.375 (1.379, 4.090)	0.00181
pT	3.078 (2.104, 4.504)	<0.00001	3.622 (2.369, 5.538)	<0.00001
pN	4.483 (2.251, 8.928)	0.00002	4.416 (2.233, 8.734)	0.00002
C stage	3.128 (2.074, 4.994)	<0.00001	3.471 (2.190, 5.500)	<0.00001
E-cad	0.175 (0.067, 0.455)	0.00035	0.149 (0.055, 0.400)	0.00016
EZH2	5.149 (2.510, 10.560)	<0.00001	5.730 (2.749, 11.943)	<0.00001

[†]Analysis was done with Cox proportional hazard regression.

HR: hazard ratio; 95% CI: 95% confidence interval.