Research Article

Cancer Prevention Research

EZH2 Promotes Malignant Phenotypes and Is a Predictor of Oral Cancer Development in Patients with Oral Leukoplakia

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Abstract

Oral leukoplakia (OL) is the most common premalignancy in the oral cavity. A small proportion of OLs progresses to oral squamous cell carcinoma (OSCC). To assess OSCC risk of OLs, we investigated the role of the transcriptional repressor enhancer of zeste homolog 2 (EZH2) in oral tumorigenesis and its clinical implication as an OSCC risk predictor. Immunohistochemistry was used to measure EZH2 expression in OLs from 76 patients, including 37 who later developed OSCC and 39 who did not. EZH2 expression was associated with clinicopathologic parameters and clinical outcomes. To determine the biological role of EZH2 in OL, EZH2 level was reduced using EZH2 siRNAs in Leuk-1 cells, its impact on cell cycle, anchorage-dependent/independent growth, and invasion was assessed. We observed strong EZH2 expression in 34 (45%), moderate expression in 26 (34%), and weak/no expression in 16 (21%) of the OLs. The higher EZH2 levels were strongly associated with dysplasia ($P < 0.001$) and OSCC development ($P < 0.0001$). Multivariate analysis indicated that EZH2 expression was the only independent factor for OSCC development ($P < 0.0001$). At 5 years after diagnosis, 80% of patients whose OLs expressed strong EZH2 developed OSCC whereas only 24% patients with moderate and none with weak/no EZH2 expression did so ($P < 0.0001$). In Leuk-1 cells, EZH2 downregulation resulted in G_1 arrest; decreased invasion capability, decreased anchorage-independent growth; downregulation of cyclin D1 and upregulation of $p15^{INK4B}$. Our data suggest that EZH2 plays an important role in OL malignant transformation and may be a biomarker in predicting OSCC development in patients with OLs. *Cancer Prev Res; 4(11); 1816–24. ©2011 AACR.*

Introduction

Oral leukoplakia (OL) represents the most common oral precancerous condition (1). It is a clinical term to describe lesions that present as a white patch and cannot be characterized clinically or histologically as any other disease. Histologically, OLs have a wide spectrum, ranging from simple hyperkeratosis to severe dysplasia or carcinoma *in situ*. Each clinical appearance or phase of OL has different transformation potential, ranging from 1% to 47% in different studies (2). Malignant transformation often occurs several years after the onset of the white plaques, but it can also occur within just few months or in decades (3, 4).

The prediction of malignant potential of OL is unreliable in current clinical practice. The histopathologic grading of dysplasia is still the most contemporary method to assess the malignant potential in patients with OL, yet this method is subjective and the clinical decisions based on the method are not satisfactory (5, 6). Considering the high morbidity and mortality associated with oral squamous cell carcinoma (OSCC), the major challenges are to identify OLs with higher risk for OSCC development independent of dysplastic changes and to reveal molecular targets that may be regulated to prevent OSCC development (7, 8).

Development of OSCC is evolutionary and characterized by multistep carcinogenic processes, in which activation of oncogenes and inactivation of tumor suppressor genes are the key features leading to clonal expansion and malignant transformation of normal oral epithelial cells (9–11). Enhancer of zeste homolog 2 (EZH2) is the catalytic subunit of Polycomb repressive complex 2 (PRC2), a highly conserved histone methyltransferase that methylates lysine 27 of histone H3 (H3-K27; ref. 12). H3-K27 methylation is commonly associated with DNA methylation and silencing of genes responsible for differentiation in organisms, ranging from plants to mammals including humans

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(12, 13). It has been shown that EZH2 is involved in methylation and silencing of a subset of genes implicated in cell differentiation, suggesting that it may play a key role in cell differentiation and maintenance of adult stem cell populations (14, 15). Furthermore, overexpression of EZH2 has been observed in several human cancer types including oral cancers, suggesting that *EZH2* is an oncogene and plays a role in oral tumorigenesis (16–18).

In this study, we tested the hypothesis that EZH2 activation is critical in malignant transformation of oral epithelial cells and therefore may serve as an indicator to predict OSCC risk in patients with OLs.

Patients and Methods

Patients and specimens

Patients were selected from an archival database on the basis of clinical diagnosis of OL from the period of 1993 through 2006 with follow-up information and available surgical biopsy samples at the time of diagnosis in the Department of Pathology and the Department of Oral and Maxillofacial Surgery, School of Stomatology, Shanghai Jiao Tong University School of Medicine, Shanghai, China. The study was reviewed and approved by the local Institutional Review Board. Thirty-seven OL patients who later developed OSCC were identified. Additional 39 OL patients who did not develop OSCC during the follow-up were selected from a larger pool of patients in the database. Paraffin tissue blocks were retrieved, and sections were made for the proposed study. One of the tissue sections from each patient was stained with hematoxylin and eosin and examined to verify histopathologic diagnosis before further analysis.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissues were cut into 4-μm tissue sections. The avidin-biotin complex (ABC) technique was used following Vectastatin elite ABC kit (Vector Laboratories). Briefly, tissue sections were deparaffinized in xylene, rehydrated in graded ethanol, treated with Tris-EDTA buffer for antigen retrieval, and quenched in hydrogen peroxide. Tissue sections were blocked with 2.5% normal serum, incubated overnight at 4°C with anti-EZH2 antibody 1:200, (clone 11; BD Transduction Laboratories), followed by biotinylated secondary antibody and then ABC reagent. Diaminobenzidine was used as chromogen and counterstained with Mayer's hematoxylin (Sigma-Aldrich Corp.). The EZH2 labeling index (LI) was defined semiquantitatively as the intensity of staining (0, 1, 2, 3), multiplied by the percentage of positive epithelial thickness (25%, 50%, 75%), and given the scores weak, moderate, and strong for the quartiles of the EZH2 LI $\leq$75, 100, and $\geq$150 to $\leq$225, respectively, as the weighted mean of cells displaying nuclear immunoreactivity (19). For comparison, the samples were also analyzed using the Aperio ScanScope and Image Scope software, Nuclear v9 algorithm, with quartiles $\leq$75, >75 to <150, and $\geq$150, as weak, moderate, and strong, respectively.

Cell culture

OL cell line (Leuk-1; ref. 20) was cultured in keratinocyte serum-free (KSF) medium with 25 μg/mL bovine pituitary extract (BPE) and 0.2 ng/mL recombinant epidermal growth factor (rEGF; Invitrogen).

siRNA knockdown

Two anti-EZH2 siRNAs, each targets the 2 splice variants of EZH2, and FAM-labeled negative control siRNA were purchased from (Ambion Inc). The anti-EZH2 siRNA sequences were 5′-GCUGACCAUUGGGACAGUATT-3′ (for siRNA-4916 or si-4916) and 5′-GUGUAUGAGUUUA-GAGUCATT-3′ (for siRNA-4917 or si-4917). *In vitro* transient transfection was done using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol.

Western blot

Cells were harvested in RIPA buffer (Sigma Aldrich). Whole-cell lysate was separated using SDS-PAGE. Primary antibodies against EZH2 (clone 11; BD Transduction Laboratories), cyclin D1, cyclin D3, $p16^{INK4A}$, $p15^{INK4B}$, $p27^{Kip1}$, CDK4, and CDK6 (Cell Signaling Biotechnology) were used. Bands Quantification was done using the Image J processing and analysis software. GAPDH antibody (Santa Cruz Biotechnology Inc.) was used to normalize protein loading.

Cell-cycle analysis

Leuk-1 cells were harvested, fixed in 70% ethanol, and suspended in PI/RNase staining buffer (BD Pharmingen) containing 0.1% sodium citrate and 0.1% Triton X-100. Data analysis was done using FlowJo software.

Cell proliferation assay

Viability of Leuk-1 cells transfected with EZH2 siRNA was measured every 24 hours for 6 days by using the Cell Proliferation Reagent WST-1 (Roche Diagnostics Corporation). The experiment was independently repeated 3 times.

Anchorage-independent growth assay

Twenty-four hours after EZH2 siRNA transfection, cells in 0.35% agarose with KSF, rEGF, and BPE medium were plated on top of solidified 0.5% agarose in 6-well plate in triplicates. The gels were covered with 1 mL medium and incubated for 3 weeks with medium change every 3 to 4 days. Colonies more than 0.1 mm in diameter were counted under a microscopic field at 40× magnification. Means of colonies were calculated on the basis of numbers from triplicate wells for each treatment condition.

In vitro cell invasion assay

BD BioCoat Matrigel invasion chambers (BD Biosciences) were used. EZH2 siRNA–transfected leuk-1 cells were seeded in the top chamber in KSF media without rEGF and BPE whereas KSF media with rEGF and BPE was placed in the bottom chamber. After 20 hours incubation, cells on the bottom surface of the membrane were fixed in 4% paraformaldehyde and stained with 0.5% crystal violet.

Cells in at least 6 random microscopic fields 100× were counted. The experiment was done in duplicates and repeated 3 times.

Statistical analysis

Event-free survival (EFS) or "OSCC-free survival" was the outcome variable. The independent variable EZH2 expression status and its interaction with histology were estimated by the Kaplan–Meier method. The log-rank test was used for univariate associations between EZH2 expression status and EFS, and individual patient characteristics and EFS. The EFS rates at years 3 and 5 with their corresponding 95% CIs were calculated. The associations between EZH2 expression status and patient characteristics were evaluated using the Fisher exact test for categorical variables and the Kruskal–Wallis test for continuous variables. The independence of each of those associations from OSCC status was evaluated using the Cochran–Mantel–Haenszel test. The Cox proportional hazards model was used for multivariate analyses. The HRs with their corresponding 95% CIs and *P* values were reported. All the analyses were conducted using SAS 9.1.3 software. All tests were 2-sided, and values of $P < 0.05$ were considered statistically significant. Paired *t* test was used for analysis of the *in vitro* studies.

Results

Patients with OL

The study cohort consisted of 76 OL patients who had no cancer history from a single hospital. Among these patients, 37 (49%) developed OSCC after the initial OL diagnosis, with median follow-up time of 2 years, whereas 39 (51%) remained OSCC free with a median follow-up time of 11.4 years. The general characteristics of the patient population are presented in Table 1 and Supplementary Table1.

Table 1. Association between EZH2 expression and patient characteristics

		EZH2 expression						
		Weak (0–1)		Moderate (2)		Strong (3)		
Characteristic	No. of patients	*n*	%	*n*	%	*n*	%	*P*
All patients	76	16	21	26	34	34	45	
Age, y								
Mean ± SD	55.1 ± 13.6	52.4 ± 11.1		52.7 ± 13.6		58.1 ± 14.3		0.24
Median	53.5	53.5		51.5		59.0		
Minimum, maximum	25.0, 82.0	28.0, 76.0		25.0, 80.0		28.0, 82.0		
Age group								
<60 y	47	11	24	18	38	18	38	0.40
≥60 y	29	5	17	8	28	16	55	
Sex								
Female	34	5	15	13	38	16	47	0.48
Male	42	11	26	13	31	18	43	
Histology								
Dysplasia	57	6	11	21	37	30	53	<0.001
Other[a]	19	10	53	5	26	4	21	
Anatomic site[b]								
Low-risk areas	31	11	35	9	29	11	35	0.04
High-risk areas	45	5	11	17	38	25	51	
Smoking status								
Yes	14	4	28	5	36	5	36	0.45
No	51	8	16	17	33	26	51	
Unknown	11	4	36	4	36	3	28	
Alcohol consumption status								
Yes	17	6	35	6	35	5	30	0.09
No	48	6	13	16	33	26	54	
Unknown	11	4	36	4	36	3	28	

NOTE: *P* value of the Kruskal–Wallis test for age as a numeric variable and that of the Fisher exact test for age group and other variables.

[a]Nondysplastic OL lesions (hyperkeratosis, acanthosis, and hyperplasia).

[b]Low-risk areas: buccal mucosa, labial mucosa, gingiva, and palate. High-risk areas: floor of mouth, lateral and ventral tongue.

EZH2 expression and clinicopathologic parameters

EZH2 expression was mainly observed as nuclear staining, whereas cytoplasmic staining was observed in some cases. The intensity of EZH2 expression in the OLs ranged from negative to strongly positive, and the extent of EZH2 expression varied from being limited only to the basal cell layer to being observed in full thickness of the epithelium (Fig. 1A).

Among the OL lesions, 34 (45%) showed strong EZH2 staining, 26 (34%) showed moderate EZH2 staining, and 16 (21%) showed no or weak EZH2 staining (Table 1). EZH2 staining levels were strongly associated with the grade of dysplasia ($P < 0.001$; Fig. 1A and Table 1). However, the expression of EZH2 was independent of dysplasia (Table 1 and Supplementary Fig. S1).

EZH2 expression and OSCC development

To determine the role of EZH2 expression in OSCC development, we analyzed OSCC-free survival probability, which was defined as years from time of diagnosis of OL to time of diagnosis of OSCC, based on EZH2 expression in OLs. We found that EZH2 expression was strongly associated with OSCC-free survival in an expression level–dependent manner ($P < 0.0001$ by log-rank test; Fig. 1B). At 5 years, none of the 16 patients whose lesions showed no or weak EZH2 expression developed OSCC whereas 6 (24%) of the moderate and 28 (80%) of the strong EZH2 expressing OLs developed OSCC. Consistent with this, digital image analysis (Supplementary Fig. S2) showed only 1 of 15 lesions with weak EZH2 expression developed OSCC after 5 years of OL diagnosis (Supplementary Table S1). In the univariate analysis, we analyzed the association between the potential risk factors including EZH2 expression and OSCC development at 3 and 5 years after OL diagnosis. EZH2 expression and OL histology were significantly associated with OSCC development ($P < 0.0001$ and $P < 0.01$, respectively; Table 2). Interestingly, alcohol use showed a borderline association with a reduced OSCC risk ($P = 0.05$). We then conducted a multivariate analysis to include EZH2 expression, OL histology, and alcohol use as cofactors. In this analysis ($N = 53$), we found that EZH2 expression was the only independent factor significantly associated with OSCC development ($P < 0.0001$; Table 3).

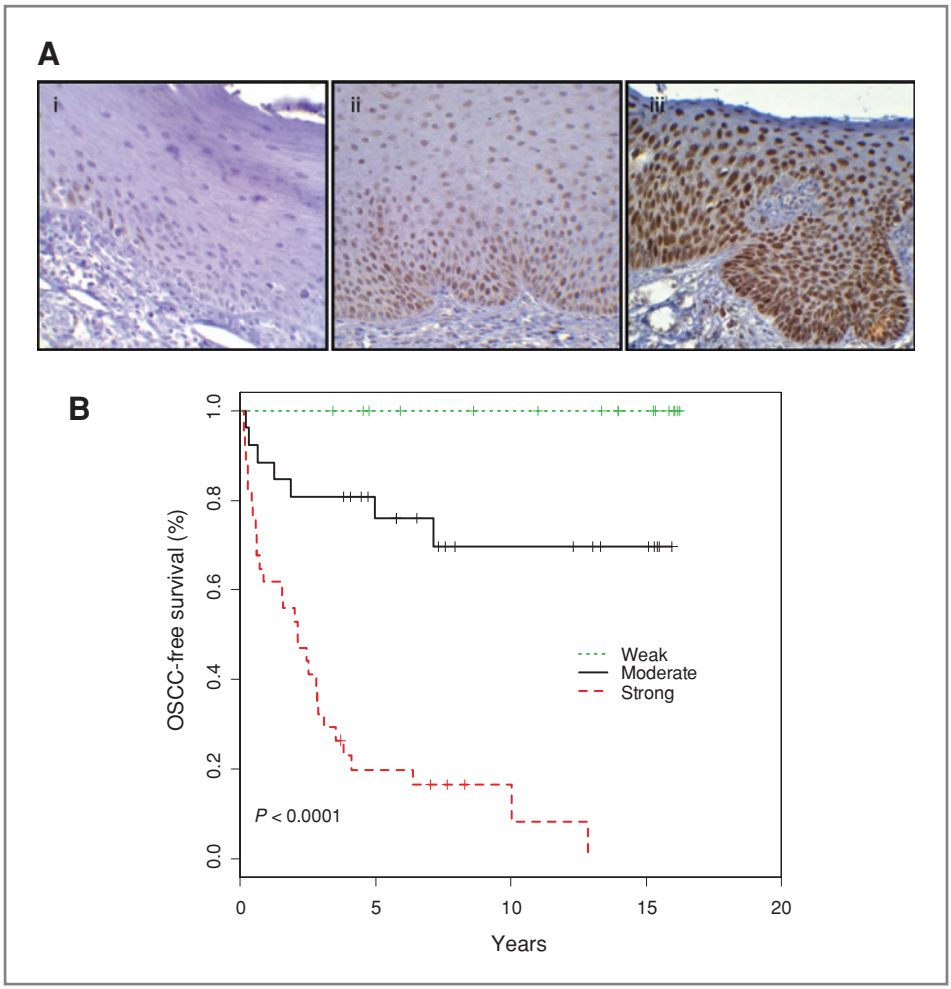

Figure 1. EZH2 expression and OSCC development. A, immunohistochemical expression of EZH2 in OL tissue samples: nuclear expression of EZH2 strongly correlated with the grade of dysplasia, the grade of dysplasia increasing from (i) weak, (ii) moderate to (iii) strong (40×). B, Kaplan–Meier curves of OSCC-free survival based on EZH2 expression.

Table 2. Three- and 5-year OSCC-free survival rates by individual risk factor ($N = 76$)

	Rate (%) ± SE		
Characteristic	**3-y EFS**	**5-y EFS**	***P***
All patients			
EZH2 expression	63 ± 6	56 ± 6	
Weak	100 ± 0	100 ± 0	<0.0001
Moderate	81 ± 8	76 ± 9	
Strong	32 ± 8	20 ± 7	
Age, y			
<60	66 ± 7	59 ± 7	0.28
≥60	59 ± 9	51 ± 9	
Sex			
Female	56 ± 9	46 ± 9	0.15
Male	69 ± 7	64 ± 7	
Histology			
Dysplasia	56 ± 7	46 ± 7	<0.01
Other	84 ± 8	84 ± 8	
Anatomic site			
Low-risk areas	68 ± 8	68 ± 8	0.17
High-risk areas	60 ± 7	48 ± 8	
Smoking status[a]			
Yes	64 ± 13	64 ± 13	0.66
No	57 ± 7	46 ± 7	
Unknown			
Alcohol status[a]			
Yes	76 ± 10	76 ± 10	0.05
No	52 ± 7	41 ± 7	
Unknown			

NOTE: *P* value of the log-rank sum test overall.
[a]There were 11 cases whose smoking status and alcohol consumption status were unknown, for which the sample size is 65 instead of 76.

Table 3. Cox proportional hazards regression models in estimating OSCC development

Characteristic	***P***	**HR**	**95% CI**
EZH2 expression			
Weak[a]	<0.0001	NA	NA
Moderate		0.17	0.07–0.41
Strong		1.00	–
Histology			
Dysplasia	0.88	1.08	0.36–3.26
Other		1.00	–
Alcohol status			
No	0.17	1.97	0.75–5.16
Yes		1.00	–

NOTE: The Cox proportional hazards regression model excluded 23 cases who either missed alcohol status or had "weak" as EZH2 expression level.
[a]The HR for the weak EZH2 expression group was not calculated because all cases in that group were censored.

EZH2 and malignant features in OL cells

To explore the underlying mechanism by which EZH2 contribute to malignant transformation of OL cells, we used EZH2 siRNAs (si-4916 and si-4917) to specifically downregulate EZH2 expression levels in an OL cell line, Leuk-1. At 72 hours after EZH2 siRNA treatment, Leuk-1 cells showed reduced EZH2 levels and proliferation. The cells also showed an increased fraction of G_1 phase (G_1 arrest) with the decreased EZH2 level in comparison with the cells treated with scrambled siRNA (Fig. 2A). In an effort to identify molecular mechanisms underlying the G_1 cell-cycle arrest by EZH2 downregulation, we measured a panel of key proteins involved in G_1 phase regulation. At 48 hours after EZH2 siRNA treatment, levels of cyclin D1 were significantly reduced whereas $p15^{INK4B}$ levels were increased (Fig. 2B). No change was observed for cyclin D3, $p16^{INK4A}$, $p27^{Kip1}$, CDK4, and CDK6 expression levels (Fig. 2B). These data suggest that EZH2 may play a role in early oral tumorigenesis by promoting cell-cycle progression through modulating $p15^{INK4B}$ and cyclin D1.

Considering that EZH2 has been associated with poor clinical outcomes in patients with OSCC (17, 18), we evaluated the effect of EZH2 in malignant transformation of Leuk-1 cells. The proliferation of Leuk-1 cells cultured on plastic surface was significantly reduced by the downregulation of EZH2 level in a dose-dependent manner compared with the cells treated with scrambled siRNA (Fig. 3A). We then analyzed the effect of EZH2 downregulation in anchorage-independent growth by using soft agar colony formation assay. Leuk-1 cells treated with EZH2 siRNAs showed significantly reduced capability to form colonies in soft agar (Fig. 3B). Because cyclin D1 has been linked to malignant progression of oral premalignancy (21, 22), we investigated the possibility that EZH2 may promote invasion of Leuk-1 cells through modulating the expression of cyclin D1. EZH2 siRNA–treated cells exhibited a significantly reduced invasion capability measured by a modified Boyden chamber invasion assay (Fig. 3C).

Discussion

A molecular-based model for OSCC development has been proposed in literature that includes deregulation of cell-cycle proteins (23). Cyclin D1 is one of the proteins that is frequently upregulated in oral premalignancies leading to malignant transformation and an increased risk of OSCC development (21, 22). Although *CCND1* gene may be amplified in oral tumorigenesis, overexpression of cyclin D1 is also observed in OLs and OSCCs without the gene amplification (21). It has been reported that high EZH2 expression is associated with upregulation of cyclin D1 in gastric cancer whereas the pharmacologic inhibition of EZH2 leads to downregulation of cyclin D1 levels in

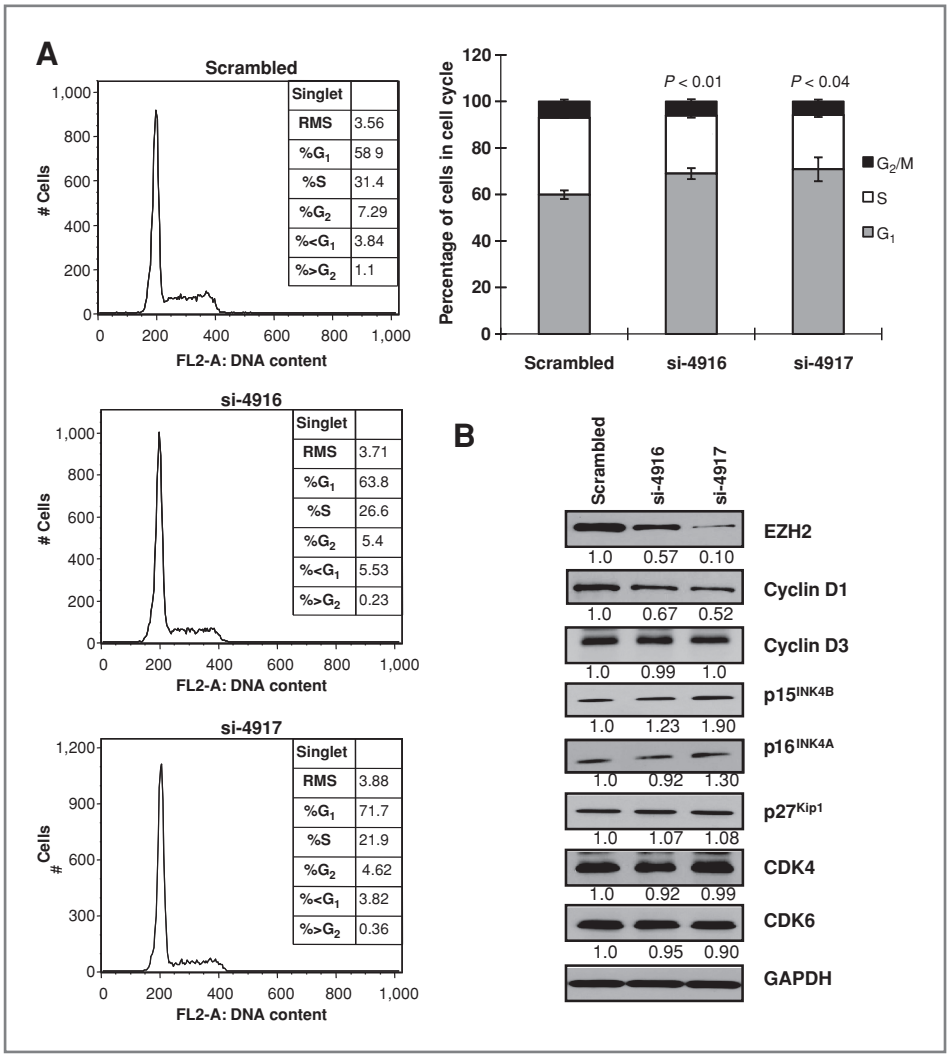

Figure 2. EZH2 and cell-cycle regulation in OL cells. A, cell-cycle analysis of Leuk-1 cells transfected with scrambled siRNA or anti-EZH2 siRNA si-4916 and si-4917 for 72 hours. B, cyclin D1 and $p15^{INK4B}$ expression in Leuk-1 cells transfected with scrambled siRNA or anti-EZH2 siRNA si-4916 and si-4917 for 48 hours.

skin cancer (24, 25). Our observation that EZH2 downregulation resulted in a decreased cyclin D1 expression level in OL cells is consistent with the previous reports and suggests that EZH2 is involved in cyclin D1 regulation in oral tumorigenesis.

In OLs and OSCC, *CDKN2B* promoter hypermethylation is frequently observed (26, 27). EZH2 may mediate this process through H3-K27 trimethylation and recruitment of DNA methyltransferases (15, 28, 29). Consistent with this mechanism, we found increased $p15^{INK4B}$ level in OL cells when EZH2 is downregulated. This provides additional support to the role EZH2 plays in oral tumorigenesis.

It is of particular interest to note that EZH2 can be activated by human papillomavirus (HPV) E7 oncoprotein in cervical tumorigenesis (30). Although the role of HPV in oral tumorigenesis has not been well established, HPV infection was casually linked to the development of oropharyngeal squamous cell carcinoma in about 40% of the patients (31), suggesting that HPV may be involved in early oral tumorigenesis (32). Thus, activation of EZH2 by HPV E7 in oral epithelial cell in oral tumorigenesis warrants further investigation.

The strong association between EZH2 expression in OLs and OSCC development is interesting and provocative. For practical reason, we analyzed OSCC incidences at 3 and 5 years after OL diagnosis to provide information about the potential impact of EZH2 as a predictive marker in OSCC risk assessment. Considering that in 80% of the patients with strong EZH2 expression OLs developed OSCC in 5 years whereas none with weak expression and 24% with moderate expression, EZH2 is one of the best single markers with potential to predict oral cancer risk development for patients with OLs ever reported in literature (18, 33). It is important to note that EZH2 expression is associated with dysplasia histology, but such association was dependent on EZH2 expression in terms of relationship with OSCC development as evidenced in our multivariate analysis (Table 3).

Contrary to most of the studies, we observed a borderline association between alcohol and tobacco use and a reduced

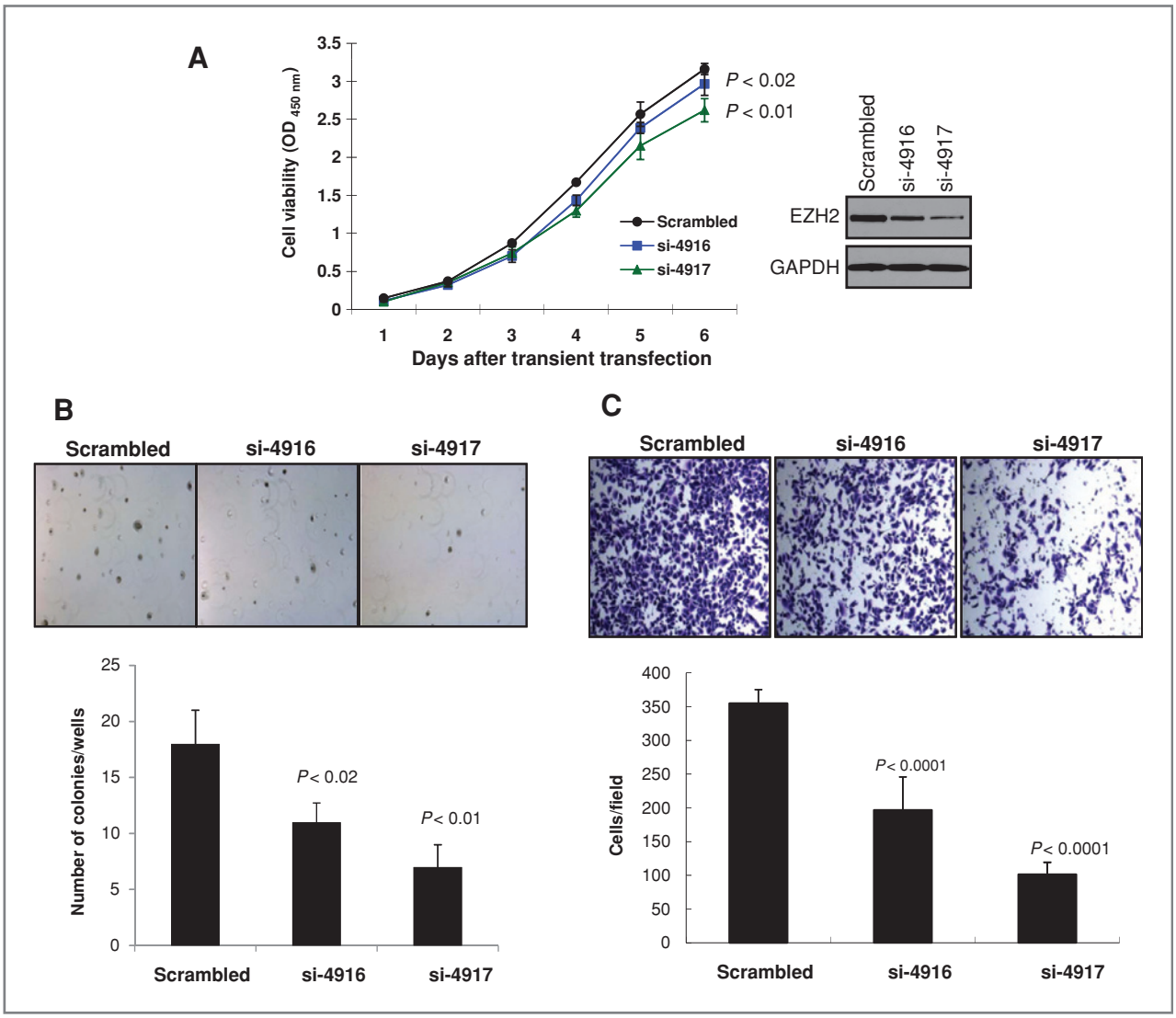

Figure 3. EZH2 promotes malignant potential of Leuk-1 cells. A, proliferation of Leuk-1 cells transfected with scrambled siRNA or anti-EZH2 siRNA si-4916 and si-4917 for 72 hours. B, colony formation of Leuk-1 cells (40×) transfected with scrambled siRNA or anti-EZH2 siRNA si-4916 and si-4917. C, invasion assay of Leuk-1 cells (100×) transfected with scrambled siRNA or anti-EZH2 siRNA si-4916 and si-4917 for 72 hours.

oral cancer risk in our patient population. Although this may be a result of the relatively small sample size, other factors might contribute to this observation. The population is from China where heavy smokers often drink liquor containing more than 50% alcohol regularly whereas the drinks consumed by most of the Westerners contain much lower concentrations of alcohol. It remains to be determined whether the higher alcohol concentration in Chinese drinks plays a different role in oral cancer development with or without tobacco exposure. Another possible contributing factor is the oral hygiene status of the population. Whether their oral microbial spectrum, which may also be influenced by smoking and drinking habits, impacts oral cancer development will need further investigation.

Sophisticated imaging equipment and software in pathologic work have gained extensive use in the past several years. A comparison between the digital image analysis and semiquantitative scoring indicates that the scores were most consistent in the weakly stained specimen but differ more in the moderately and strongly stained specimen. Human semiquantitative analysis clearly had the tendency to give more polarized score than the digital image analysis (Supplementary Table S2). Importantly, despite the difference, the general conclusion in this study did not change.

The current study has several limitations. First, it is a retrospective case–control study that artificially enriched the proportion of patients who later developed OSCC and therefore may overestimate the predictive value of the biomarker. Second, the study is based on a Chinese

population in a single hospital. Considering various genetic and environmental factors that can contribute to oral tumorigenesis, our findings in this population may have a limited implication in other populations at different geographic locations with various genetic backgrounds. Therefore, additional studies are necessary to validate these findings by using samples obtained prospectively in different geographic locations and from populations with various genetic backgrounds.

Nevertheless, in this study, we identified, for the first time, that EZH2 expression is an independent predictor for OSCC development in patients with OLs and provided evidence to support the biological link between EZH2 and cell proliferation and invasion in OL cells. If validated in future studies, EZH2 may serve as a biomarker for oral cancer risk assessment of patients with OLs and a potential target for oral cancer chemoprevention (34).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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