Up-Regulation of Enhancer of Zeste Homolog 2 Is Associated Positively With Cyclin D1 Overexpression and Poor Clinical Outcome in Head and Neck Squamous Cell Carcinoma

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BACKGROUND: The authors previously observed that enhancer of zeste homolog 2 (EZH2) overexpression was associated significantly with the development of oral cancer. In the current study, they investigated whether EZH2 can function as a prognostic predictor for patients with head and neck squamous cell carcinoma (HNSCC). METHODS: Expression levels of EZH2 in HNSCC cells were detected using reverse transcriptase-polymerase chain reaction (PCR) and Western blot analyses. In addition, the effects of EZH2 ablation on the proliferation and invasion of HNSCC cells were investigated through small interfering RNA (siRNA)-mediated knockdown. Real-time PCR and immunohistochemistry were used to evaluate EZH2 and cyclin D1 expression in 46 HNSCC samples, and the expression levels also were re-evaluated in 124 independent samples by immunohistochemistry. RESULTS: EZH2 expression was elevated remarkably in HNSCC specimens and cell lines. Upon EZH2 silencing, the proliferation and invasion of HNSCC cells were remarkably suppressed. EZH2 expression frequently was correlated with cyclin D1 expression (P = .034) and tumor differentiation (P = .020). In addition, both EZH2 messenger RNA levels and EZH2 protein levels were strongly associated with signs of histologic severity (P = .012 and P = .032, respectively). Univariate analysis revealed that high EZH2 expression was associated with worse overall survival (P = .001) and disease-free survival (P = .002). The combined expression of EZH2 and cyclin D1 had superior prognostic ability for patients with HNSCC than the expression of either marker alone. In multivariate analysis, EZH2 expression was identified as an independent predictor of overall and disease-free survival. CONCLUSIONS: The current results indicated that EZH2 is an independent prognostic indicator for patients with HNSCC. In addition, an analysis of the combined expression of EZH2 and cyclin D1 can serve as a more powerful prognostic predictor for patients with HNSCC. Cancer 2012;118:2858-71. © 2011 American Cancer Society.

KEYWORDS: enhancer of zeste homolog 2, cyclin D1, head and neck, squamous cell carcinoma, clinical outcome, prognostic predictor.

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC), the sixth most common cancer in the world, is widely represented as a heterogeneous tumor with more aggressive phenotypes. However, despite ongoing efforts over the past 3 decades, traditional treatments (surgery, radiotherapy, and chemotherapy) have not sufficiently improved the 5-year survival rate of patients with these devastating diseases, especially those with advanced head and neck cancer who have locoregional lymph node metastases. This mostly likely is because of the absence of a well established understanding of the molecular basis of HNSCC initiation and a lack of biomarker identification in HNSCC progression. However, it is well known that the development of HNSCC is an evolutionary process and is characterized by multistep carcinogenic processes in which activation of oncogenes and inactivation of tumor suppressor genes, including tumor protein 53 (TP53), epidermal growth factor receptor
(EGFR), cyclin D1 (CCND1), and cyclin-dependent kinase inhibitor 2A (CDKN2A), are caused by genetic alterations and epigenetic modifications.2-5 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).6 H3-K27 trimethylation is commonly associated with DNA methylation and the silencing of genes responsible for differentiation in organisms ranging from plants to mammals, including humans.6,7 It has been demonstrated 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.8,9 Phosphorylation of threonine 350 (Thr350) and phosphorylation of Thr487 in EZH2 are evolutionarily conserved motifs that recently were identified by mass spectrometry and were identified as important for the recruitment of EZH2 and maintenance of H3-K27me3 levels at EZH2 target loci.10-12 Recent studies have demonstrated that EZH2 is frequently overexpressed in several human epithelial cancer types, including prostate cancer, inflammatory breast cancer, gastric cancer, nonsmall cell lung cancer, esophageal cancer, salivary adenoid cystic carcinoma, recurrent nasopharyngeal carcinoma, and oral cancer, and it has been linked to a poor prognosis in patients with those cancers.13-20 In addition, in our prior study, we observed that high EZH2 levels frequently were correlated with oral cancer development in patients with oral leukoplakia.21 Together, the previous studies suggest that EZH2 may be a key oncoprotein involved in tumor initiation and progression. In the current study, we provide evidence that EZH2 notably correlates with cyclin D1 oncoprotein expression, serving as a critical predictor of more aggressive tumor phenotypes, and can be used to determine the prognosis of patients with HNSCC. Our findings demonstrate that the up-regulation of EZH2 is frequent in HNSCC specimens and cells; furthermore, EZH2 has a biologic role that affects the proliferation and invasion of HNSCC cells. High EZH2 expression notably was associated with cyclin D1 expression, poorly differentiated tumors, more severe histologic signs of HNSCC, and worse overall and disease-free survival. EZH2 and cyclin D1 levels together may be a superior predictor of prognosis in patients with HNSCC than the levels of each marker alone. The results suggest that EZH2, together with cyclin D1, may be a novel and promising prognostic predictor for patients with HNSCC.

MATERIALS AND METHODS

Cell Culture

The human head and neck squamous cell carcinoma cell lines WSU-HN4, HN6, HN12, HN13, and HN30 (kindly provided by the University of Maryland Dental School) were cultured in Dulbecco modified Eagle medium (DMEM) (GIBCO-BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GIBCO-BRL) penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37°C in a humidified 5% CO2 atmosphere. SCC-4, SCC-9, and SCC-25 cells (purchased from the American Type Culture Collection, Manassas, Va) were cultured in DMEM/F12 medium (GIBCO-BRL) supplemented with 10% heat-inactivated FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL). Then, immortalized oral epithelial cells infected with human papillomavirus type 16-E6E7 (HPV16E6E7) (established in the Ninth People’s Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China) were cultured in a defined keratinocyte serum-free medium (GIBCO-BRL).

Patients and Specimens

Samples from a cohort of 46 patients who were diagnosed with primary head and neck squamous cell carcinoma between June 2008 and July 2009 were collected. All samples were obtained by surgery. Half of each sample was quickly frozen in liquid nitrogen until total RNA was extracted, and the other half was embedded in paraffin for pathologic examination. The study cohort comprised 17 patients with clinical stage I/II disease and 29 patients with stage III/IV disease. The patients underwent initial surgical treatment at the Department of Oral and Maxillofacial Surgery, School of Stomatology, Shanghai Jiao Tong University School of Medicine (Shanghai, China). The 46 patients with HNSCC included 30 men (65.2%) and 16 women (34.8%) with a median age of 59.5 years (Table 1). In parallel, a separate cohort of 124 patients also was assembled from a large pool of patients in the database based on histologic diagnosis of HNSCC who had undergone radical surgery in the Department of Oral and Maxillofacial Surgery, School of Stomatology, Shanghai Jiao Tong University School of Medicine (Shanghai, China) between 1998 and 2004. Exclusion criteria included recurrence at presentation, preoperative radiotherapy, chemotherapy, clinical stage I/II, distant metastasis, and incomplete medical records. We retrospectively reviewed the medical records of patients with HNSCC according to the inclusion and exclusion criteria. This cohort of patients with locally advanced HNSCC
Table 1. Associations Between EZH2 Messenger RNA Levels and Clinical Parameters (N = 46)

| Characteristic                        | No. of Patients | EZH2 ΔCtT (Tumor)ᵃ |  | EZH2 ΔCtN (Normal)ᵃ |  | EZH2 ΔCtT (Tumor)/EZH2 ΔCtN (Normal)ᵃ |
|---------------------------------------|----------------|---------------------|---|---------------------|---|--------------------------------------|
|                                       |                | Mean ± SD           | P | Mean ± SD           | P | Mean ± SD                             |
| Age, y                                |                |                     |   |                     |   |                                      |
| ≥60                                   | 23             | 7.45 ± 1.52         | .416 | 8.12 ± 1.33       | .244 | 0.94 ± 0.24                      | .590 |
| <60                                   | 23             | 7.84 ± 1.03         | .610 | 8.70 ± 1.40       | .213 | 0.92 ± 0.16                      | .872 |
| Sex                                   |                |                     |   |                     |   |                                      |
| Men                                   | 30             | 7.76 ± 1.38         | .213 | 8.54 ± 1.39       | .704 | 0.92 ± 0.20                      | .872 |
| Women                                 | 16             | 7.43 ± 1.14         | .940 | 8.17 ± 1.38       | .940 | 0.94 ± 0.21                      | .872 |
| Smoking history                       |                |                     |   |                     |   |                                      |
| Smoker                                | 13             | 7.29 ± 1.63         | .487 | 7.88 ± 0.73       | .102 | 0.93 ± 0.20                      | .55  |
| Nonsmoker                             | 33             | 7.79 ± 1.14         | .940 | 8.62 ± 1.53       | .940 | 0.92 ± 0.21                      | .872 |
| Alcohol history                       |                |                     |   |                     |   |                                      |
| Drinker                               | 8              | 7.91 ± 0.56         | .505 | 7.98 ± 0.82       | .361 | 0.91 ± 0.21                      | .192 |
| Nondrinker                            | 38             | 7.59 ± 1.41         | .940 | 8.50 ± 1.46       | .940 | 1.00 ± 0.10                      | .872 |
| Tumor grade                           |                |                     |   |                     |   |                                      |
| Well differentiated                   | 16             | 8.04 ± 0.72         | .103 | 8.25 ± 1.43       | .337 | 1.00 ± 0.16                      | .777 |
| Moderately to poorly differentiated   | 30             | 7.46 ± 1.48         | .940 | 8.49 ± 1.38       | .940 | 0.90 ± 0.21                      | .872 |
| TNM stage                             |                |                     |   |                     |   |                                      |
| I                                      | 9              | 8.18 ± 1.12         | .428 | 8.78 ± 1.64       | .832 | 0.97 ± 0.23                      | .751 |
| II                                     | 8              | 7.50 ± 0.59         | .940 | 8.07 ± 0.98       | .940 | 0.95 ± 0.16                      | .872 |
| III                                    | 12             | 7.42 ± 1.83         | .940 | 8.64 ± 1.53       | .940 | 0.87 ± 0.24                      | .872 |
| IV                                     | 17             | 7.59 ± 1.21         | .940 | 8.21 ± 1.34       | .940 | 0.94 ± 0.18                      | .872 |
| Lymph node metastasis                 |                |                     |   |                     |   |                                      |
| pN0                                    | 35             | 7.62 ± 1.34         | .807 | 8.48 ± 1.35       | .787 | 0.94 ± 0.22                      | .291 |
| pN1-pN2                               | 11             | 7.75 ± 1.22         | .940 | 8.17 ± 1.53       | .940 | 0.92 ± 0.18                      | .872 |
| Disease site                          |                |                     |   |                     |   |                                      |
| Tongue                                | 22             | 7.46 ± 0.94         | .147 | 8.52 ± 1.15       | .494 | 0.89 ± 0.18                      | .404 |
| Gingiva                               | 5              | 7.26 ± 2.88         | .940 | 8.35 ± 0.86       | .940 | 0.98 ± 0.20                      | .291 |
| Buccal mucosa                         | 14             | 8.07 ± 1.17         | .940 | 8.55 ± 1.96       | .940 | 0.85 ± 0.32                      | .872 |
| Floor of mouth                        | 2              | 8.42 ± 0.41         | .940 | 7.96 ± 0.36       | .940 | 1.06 ± 0.10                      | .872 |
| Hard palate                           | 3              | 7.23 ± 0.57         | .940 | 7.34 ± 0.58       | .940 | 0.99 ± 0.15                      | .872 |
| Histologic signs of severity          |                |                     |   |                     |   |                                      |
| Present                               | 23             | 7.60 ± 1.02         | .177 | 8.70 ± 1.32       | .110 | 0.89 ± 0.03                      | .032 |
| Absent                                | 21             | 7.67 ± 1.55         | .940 | 8.03 ± 1.37       | .940 | 0.98 ± 0.23                      | .872 |
| Missing                               | 2              | 7.93 ± 2.16         | .940 | 8.99 ± 2.11       | .940 | 0.98 ± 0.03                      | .872 |

Abbreviations: EZH2, enhancer of zeste homolog 2; SD, standard deviation; TNM stage, tumor-node-metastasis stage.
ᵃΔCt indicates the difference in the cycle number at which a sample’s fluorescent signal passes a given threshold above baseline (Ct) derived from a specific gene compared with that of β-actin in tumor tissue (ΔCt [Tumor]) and in normal tissue (ΔCtN [Normal]).

Included 89 men (71.8%) and 35 women (28.2%) with a median age of 57 years, and their clinicopathologic characteristics are summarized in Table 2. The median follow-up was 102 months (interquartile range, 78-110 months). In this study, tumors from each patient were stained with hematoxylin and eosin (H&E), classified histologically, and staged according to the International Union Against Cancer tumor-lymph node-metastasis (TNM) classification system before further analysis.

Semiquantitative Reverse Transcriptase-Polymerase Chain Reaction Analysis

By using a reverse transcription kit (Promega, Madison, Wis), 2 μg total RNA were reverse transcribed directly to combinational DNA (cDNA) according to the manufacturer’s instructions in a total volume of 25 μL. The primer sequences used were as follows: for EZH2, the forward primer was 5’-GAA TGG AAA CAG CGA AGG ATA C-3’, and the reverse was 5’-GGG ATG ACT TGT TGT GTT-3’.
Table 2. Associations Between Enhancer of Zeste Homolog 2 Protein Levels and Clinical Parameters (N = 124)

| Characteristic                  | Total, N = 124 | EZH2 Expression, N = 117<sup>a</sup> | P       |
|--------------------------------|----------------|--------------------------------------|---------|
|                                | <Median:         | ≥Median:                             |         |
|                                | Negative         | Positive                             |         |
| Age, y                         | Mean±SD          | 57.1±12.3                            | .461    |
|                                | Median           | 57                                   |         |
|                                | Range            | 26-83                                |         |
| Sex                            |                 |                                      | .100    |
|                                | Men              | 89 (71.8)                            |         |
|                                | Women            | 35 (28.2)                            |         |
| Smoking history                |                 |                                      | .849    |
| Smoker                         |                 | 60 (48.4)                            |         |
| Nonsmoker                      |                 | 56 (45.2)                            |         |
| Missing                        |                 | 8 (6.4)                              |         |
| Alcohol history                |                 |                                      | 1.000   |
| Drinker                        |                 | 81 (65.3)                            |         |
| Nondrinker                     |                 | 35 (28.2)                            |         |
| Missing                        |                 | 8 (6.4)                              |         |
| Tumor grade                    |                 |                                      | .020    |
| Well differentiated            |                 | 96 (77.4)                            |         |
| Moderately differentiated      |                 | 25 (20.2)                            |         |
| Poorly differentiated          |                 | 3 (2.4)                              |         |
| TNM stage                      |                 |                                      | .341    |
| III                            |                 | 47 (37.9)                            |         |
| IVA                            |                 | 77 (62.1)                            |         |
| Lymph node metastasis          |                 |                                      | .564    |
| pN0                            |                 | 81 (65.3)                            |         |
| pN1-pN2                        |                 | 43 (34.7)                            |         |
| Disease site                   |                 |                                      | .425    |
| Tongue                         |                 | 48 (38.7)                            |         |
| Gingiva                        |                 | 25 (20.2)                            |         |
| Buccal mucosa                  |                 | 24 (19.4)                            |         |
| Floor of mouth                 |                 | 10 (8.1)                             |         |
| Oropharynx                     |                 | 11 (8.9)                             |         |
| Hard palate                    |                 | 4 (3.2)                              |         |
| Nasal sinuses                  |                 | 2 (1.6)                              |         |
| Histologic signs of severity   |                 |                                      | .012    |
| Present                        |                 | 69 (55.6)                            |         |
| Absent                         |                 | 50 (40.3)                            |         |
| Missing                        |                 | 5 (4)                                |         |
| HPV/p16 status<sup>b</sup>     |                 |                                      |         |
| HPV-hr                         |                 | 1 (8.1)                              | .400    |
| HPV−                           |                 | 10 (80.9)                            |         |
| p16+                           |                 | 5 (45.5)                             | .524    |
| p16−                           |                 | 6 (54.5)                             |         |
| HPV-hr/p16+                    |                 | 0 (0)                                | .333    |
| HPV-hr/p16−                    |                 | 1 (9.1)                              |         |
| HPV−/p16+                      |                 | 5 (45.5)                             |         |
| HPV−/p16−                      |                 | 5 (45.5)                             |         |

Abbreviations: +, positive; −, negative; EZH2, enhancer of zeste homolog 2; HPV-hr, high-risk human papillomavirus; p16, tumor protein 16; pN, pathologic lymph node status; TNM stage, tumor lymph node metastasis stage.

<sup>a</sup>Expression levels of EZH2 were unavailable in 7 patients because the sections had insufficient tumor cell numbers for evaluation.

<sup>b</sup>HPV/p16 status was evaluated only in patients with oropharyngeal squamous cell carcinoma.
GGAAAAT-3'; and, for β-actin, the forward primer was 5'-TCACCCACACTGTGCCCCATCCTACGA-3', and the reverse primer was 5'-GGGATGACCTTTGTTGGAAAAT-3'. Each primer was added at a final concentration of 0.5 μM to a 15-μL reaction mixture in polymerase chain reaction (PCR) buffer that contained 1 μL cDNA, 0.25 mM of each dinucleotide triphosphate, 1.5 mM MgCl₂, and 2.5 U Taq DNA polymerase. An initial denaturation step was performed for 5 minutes at 94°C, and 35 cycles were performed with the following PCR program: denaturing at 94°C for 30 seconds, annealing at 55°C for 30 seconds for EZH2 either or β-actin, and elongation at 72°C for 30 seconds. The program was completed with a final extension at 72°C for 5 minutes. Ethidium bromide-stained bands were observed using ultraviolet transillumination, and fluorescence intensity was quantified using the FR-200 system (FuRi, Shanghai, China). The data from semiquantitative PCR reactions were normalized against the expression of β-actin from 3 independent experiments ± standard deviation. All reverse transcription-PCR (RT-PCR) data were from at least 3 independent experiments.

**Real-Time Polymerase Chain Reaction Analysis**

All real-time PCR reactions were performed using an ABI 7300 real-time PCR system (Applied Biosystems, Carlsbad, Calif) and the SYBR Premix Ex Taq reagent kit (Takara Bio Inc., Shiga, Japan). Real-time PCR analysis was performed in a final volume of 15 μL with 1.5 μL of template cDNA at a concentration of 20 ng/μL with 7.5 μL SYBR green I fluorescent dye and 10 pM of each primer for the target gene and β-actin. The primer sequences were sense 5'-TTGTTGTGGCCGCAAGCCTGTG-TAAAATC-3' and antisense 5'-TCCCTAGTCCCAGGC-CAATGACG-3' for EZH2, sense 5'-GCCCGAGGAGCTGCTGCAAA-3' and antisense 5'-TGCCACCATGGAGGGCGGAT-3' for CCND1, and sense 5'-CCTGGACCCCCAGCACAT-3' and antisense 5'-GGGC CGGACTCGTCATCT-3' for β-actin. The results of real-time PCR are represented as Ct values, where Ct is a fraction defined as the cycle number at which the sample's fluorescent signal passes a given threshold above baseline. \( \Delta Ct \) is the difference in the Ct values derived from the specific genes compared with β-actin. \( \Delta Ct \) (Tumor) = Ct target gene in tumor tissue – Ct β-actin in tumor tissue, whereas \( \Delta Ct \) (Normal) = Ct target gene in normal tissue – Ct β-actin in normal tissue. Relative messenger RNA (mRNA) expression levels of EZH2 normalized to β-actin were represented as –ΔCt value in our samples. Therefore, the higher ΔCt value was the lower relative mRNA expression level of EZH2. The significance level was set at \( P < .05 \).

**Western Blot Analysis**

Cells were harvested in radioimmunoprecipitation assay buffer (Sigma Aldrich, St. Louis, Mo). Cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Primary antibodies against EZH2 (clone 11; BD Transduction Laboratories, San Jose, Calif) were used, and glyceraldehyde 3-phosphate dehydrogenase antibody (Santa Cruz Biotechnology Inc., Santa Cruz, Calif) was used to normalize protein loading. Bands were detected using an IRDye800-conjugated affinity-purified antimouse immunoglobulin M antibody (Rockland, Gilbertsville, Pa). The membrane was then washed several times and scanned using the Odyssey infrared imaging system (LICOR, Lincoln, Neb) at an 800-channel wavelength and analyzed with Odyssey software.

**Small Interfering RNA Transfection**

Two anti-EZH2 small interfering RNAs (siRNAs) targeting the 2 splice variants of EZH2 were purchased from Ambion Inc. (Austin, Tex). FAM-labeled negative control siRNA was purchased from Shanghai Genepharmaceutical Company, Ltd. (Shanghai, China) The siRNA sequences used were 5'-GCUGACCCAUUGGGACAGUATT-3' and 5'-UACUGUCCAAUUGGUCACGGG-3' (siRNA-4916); 5'-GUGUAUGAGGUUAGACUATT-3' and 5'-UGACUCUAAACUCAUACACCT-3' (siRNA-4917); and 5'-UUCUCGAGUGUGACGATT-3' and 5'-ACGUGACACGUUCGGAGAATT-3' (negative control siRNA). In vitro transient transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, Calif) following the manufacturer’s protocol.

**Cell Proliferation Assay**

A cell-proliferation assay was performed to analyze the proliferation potential of transiently transfected EZH2 siRNAs compared with negative control siRNA in WSU-HN6 and WSU-HN13 cells. The procedure was performed as described in our previous report. Briefly, the cells were harvested and plated in 96-well plates at 1 × 10³ cells per well and maintained at 37°C in a humidified incubator. At the indicated time points, 10 μL of the CCK-8 solution were added into the triplicate wells and incubated for 1 hour, and the absorbance at 450 nm was measured to calculate the number of vital cells in each well. Measurements were performed in triplicate, and the mean ± standard deviation optical density was reported.
**Cell Invasion Assay**

An in vitro invasion assay was performed to analyze the invasive potential of WSU-HN6 and WSU-HN13 cells transfected with EZH2 siRNA or negative control siRNA. The procedure was performed as described in our previous report. Briefly, in total, $5 \times 10^5$ cells in 750 µL of serum-free DMEM were plated onto BD BioCoat Matrigel Invasion Chambers (8 µm pore size; BD Biosciences, San Jose, Calif) with the lower chamber containing 750 µL of DMEM and 10% FBS as a chemoattractant. After 24 hours of incubation in a humidified atmosphere with 5% CO₂ at 37°C, the noninvading cells were removed from the upper surface of the membrane by using a cotton swab. The membranes were then fixed with methanol and stained with 0.5% crystal violet stain. Invading cells were photographed and counted in 5 random, nonoverlapping ×200 fields under a light microscope.

**Immunohistochemical Staining**

Formalin-fixed, paraffin-embedded tissue samples were cut into 4 µm tissue sections. The avidin-biotin complex (ABC) technique was performed following the manufacturer’s instructions for the Vectastatin Elite ABC kit (Vector Laboratories, Burlingame, Calif). Briefly, tissue sections were deparaffinized in xylene, rehydrated in graded ethanol, treated with Tris-ethylene diamine tetra-acetic acid buffer or citrate 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 dilution; clone 11; BD Transduction Laboratories, San Jose, Calif) and anti-cyclin D1 antibody (1:200 dilution; clone EPR2241; Epitomics Inc., Burlingame, Calif) followed by incubation with biotinylated secondary antibody and then ABC reagent. Diaminobenzidine was used as chromogen, and sections were counterstained with Mayer hematoxylin (Sigma-Aldrich Corp., St. Louis, Mo). The labeling index was defined semiquantitatively as the intensity of staining (0, 1, 2, or 3) multiplied by the percentage of positive epithelial thickness (25%, 50%, or 75%). A pathologist randomly examined 7 to 10 tumor cell areas in each section, and a cutoff value was set as the median of the labeling index. Of the 46 patients with primary HNSCC, the pathologist excluded 9 patients because of an insufficient number of tumor cells in the sections for evaluation. Of the additional 124 patients, 7 patients similarly were excluded because of an insufficient number of tumor cells in the sections. For EZH2 staining, the cutoff value was 135; thus, a value $\geq 135$ was considered high expression, and a value $< 135$ was considered low expression. For cyclin D1 staining, the cutoff value was 110; thus, a value $\geq 110$ was considered high expression, and a value $< 110$ was considered low expression.

**Statistical Analysis**

For immunohistochemical analysis, the associations between EZH2 expression levels and patient characteristics were evaluated using Fisher exact tests for categorical variables and Kruskal-Wallis tests for continuous variables. The log-rank test was used to analyze univariate associations between expression levels of EZH2, cyclin D1, and EZH2 plus cyclin D1 and overall and disease-free survival. Then, all potential prognostic factors with P values $< .05$ from the univariate analysis were incorporated into multivariate analyses. The hazard ratios with corresponding 95% confidence intervals (CIs) and P values are reported. The correlation between levels of EZH2 expression and cyclin D1 expression was analyzed using the Pearson chi-square test. For real-time PCR analysis, the associations between EZH2 mRNA levels and patient characteristics were evaluated using the Kruskal-Wallis test. The association between EZH2 mRNA and cyclin D1 mRNA was analyzed by using Pearson correlation analysis. All analyses were conducted using the SPSS software program (standard version 13.0; SPSS Inc., Chicago, Ill). All tests were 2-sided, and P values $< .05$ were considered statistically significant. The t test for paired data was used for analysis of the in vitro studies.

**RESULTS**

**EZH2 Is Up-Regulated in Head and Neck Squamous Cell Carcinoma Tissues and Cell Lines**

To evaluate EZH2 expression patterns in HNSCC tissues and HNSCC cell lines, real-time PCR and semiquantitative RT-PCR, respectively, were performed to determine the EZH2 mRNA level in 46 paired HNSCC tissues, 9 HNSCC cell lines, immortalized head and neck epithelial cells, and normal epithelia cells (Fig. 1A). EZH2 mRNA levels were elevated in HNSCC tissues compared with the levels adjacent normal tissues ($P = .019$) (Fig. 1D). Meanwhile, EZH2 transcription levels also were increased in the 9 HNSCC cell lines and in the immortalized head and neck epithelial cells relative to normal epithelia cells. Correspondingly, we observed that EZH2 translation levels were elevated in 4 of 5 (85%) HNSCC tissues that were selected randomly from the 46 paired HNSCC
tissues and all HNSCC cell lines compared with the levels in paired normal tissues and normal epithelial cells, respectively, using Western blot analysis (Fig. 1C). Furthermore, EZH2 nuclear sublocalization was identified clearly in HNSCC cells, as illustrated in Figure 1B.

Silencing EZH2 Inhibits Head and Neck Squamous Cell Carcinoma Cell Proliferation and Invasion

Although we identified overexpression of EZH2 in HNSCC cells, its role in regulating HNSCC cancer cell proliferation is unknown. To address this issue, we silenced EZH2 using siRNAs and evaluated the ability of cells to proliferate using a cell-counting kit (CCK)-8 and Trypan blue staining (Fig. 2A). We observed that transfection of 2 EZH2 siRNAs in WSU-HN6 and WSU-HN13 cells resulted in decreased proliferation in these cells compared with the proliferation in cells that were transfected with a negative control siRNA. This suggests that EZH2 is involved in the regulation of growth signaling cascades, which positively influence HNSCC cell proliferation. Because there was no biologic link underlying EZH2 and HNSCC progression, in vitro invasion assays were performed to determine the effect of EZH2 on cell invasion using BD BioCoat Matrigel Invasion Chambers. The Matrigel matrix served as a reconstituted basement membrane in vitro. We observed that depletion of EZH2 leads to a decrease in the invasion ability of WSU-HN6 and WSU-HN13 cells, indicating that EZH2 participates in HNSCC development and progression, as illustrated in Figure 2B.

Elevated EZH2 Levels Are Strongly Associated With Cyclin D1 Overexpression

High EZH2 expression is correlated with up-regulation of cyclin D1 in gastric cancer, whereas the pharmacologic inhibition of EZH2 leads to down-regulation of cyclin D1 levels in skin cancer.24,25 However, the correlation between EZH2 expression and cyclin D1 expression has not been well established in a cohort of clinical specimens. Here, we evaluated the correlation between EZH2 and cyclin D1 transcriptional and translational levels in specimens from 46 patients with HNSCC by using real-time PCR and immunohistochemistry. It is noteworthy that a significant association between EZH2 and cyclin D1 translational levels was
observed in specimens from 46 patients with HNSCC (Pearson correlation coefficient $r = 0.385; P = .020$) (Fig. 3B); however, no significant correlation was observed between $\text{EZH2}$ and $\text{cyclin D1}$ transcriptional levels in the same cohort (Pearson $r = 0.059; P = .699$) (Fig. 3A). To validate our findings further, we re-evaluated $\text{EZH2}$ and $\text{cyclin D1}$ protein levels in an additional 124 patients with HNSCC by immunohistochemistry. The results indicated that up-regulation of $\text{EZH2}$ is correlated remarkably with $\text{cyclin D1}$ overexpression ($P = .034$) (Fig. 3C).

**Correlation Between $\text{EZH2}$ and Clinicopathologic Parameters**

By using real-time PCR for $\text{EZH2}$ mRNA in 46 patients, we observed that the $\text{EZH2} \Delta \text{CtT (Tumor)} / \Delta \text{CtN (Normal)}$ ratio was strongly associated with histologic signs of severity, including vascular embolization, perineural invasion, and diffuse invasion ($P = .032$), but not with tumor grade ($P = .077$) (Table 1) or the $\text{cyclin D1} \Delta \text{CtT (Tumor)} / \Delta \text{CtN (Normal)}$ ratio ($P = .525$), which is partially consistent with the immunohistochemical results. We established that, the lower the $\Delta \text{CtT (Tumor)} / \Delta \text{CtN (Normal)}$ ratio, the higher the $\text{EZH2}$ mRNA levels in tumor tissues, suggesting that high $\text{EZH2}$ mRNA levels are linked to histologic signs of severity. In addition, using immunohistochemical staining for $\text{EZH2}$ expression levels, we observed that nuclear staining of $\text{EZH2}$ was detected in most HNSCC specimens with diverse expression patterns, whereas no nuclear staining of $\text{EZH2}$ was observed in normal head and neck epithelial tissues (representative immunohistochemical images are provided in Fig. 4). High expression of $\text{EZH2}$ frequently was observed...
in poorly differentiated tumors ($P = .020$) with histologic signs of severity, including vascular embolization, perineural invasion, and diffuse invasion ($P = .012$) (Table 2). There were no significant associations between EZH2 expression pattern and sex ($P = .100$), smoking history ($P = .849$), alcohol history ($P = 1.000$), TNM classification ($P = .341$), lymph node metastasis ($P = .564$), or anatomic site ($P = .425$).

### Association Between EZH2 Protein Level and Clinical Outcome in Patients With Head and Neck Squamous Cell Carcinoma

To determine the correlation between EZH2 expression and clinical outcome, overall survival and the disease-free survival probability were estimated by using Kaplan-Meier survival analysis. We observed that patients whose primary tumors exhibited a high level of EZH2 (the mean
labeling index of 135 was used as a cutoff point) had significantly poorer overall survival ($P = .001$) and disease-free survival ($P = .002$) (Fig. 5A,B). In addition, our findings indicated that high cyclin D1 expression was associated significantly with worse overall survival ($P = .025$) and disease-free survival ($P = .002$) (Fig. 5C,D). Considering the correlation between EZH2 expression and cyclin D1 expression, we used EZH2 and cyclin D1 expression together to estimate the survival probability. Notably, patients who had high EZH2 expression and high cyclin D1 expression had remarkably lower rates of overall survival ($P < .001$) and disease-free survival ($P < .001$) compared with patients who had low EZH2 expression and low cyclin D1 expression (Fig. 5E,F). In univariate and multivariate Cox proportional analyses (Table 3), EZH2 expression status (hazard ratio, 1.863; 95% CI, 1.090-3.093; $P = .022$), together with histologic signs of severity (hazard ratio, 2.071; 95% CI, 1.186-3.617; $P = .010$) and lymph node metastasis (hazard ratio, 1.886; 95% CI, 1.139-3.122; $P = .014$), were identified as independent predictors of overall survival in patients with HNSCC. EZH2 expression status (hazard ratio, 1.727; 95% CI, 1.045-2.856; $P = .033$) was a more striking independent predictor than lymph node metastasis (hazard ratio, 1.658; 95% CI, 1.017-2.703; $P = .043$) or histologic signs of severity (hazard ratio, 1.632; 95% CI, 0.972-2.740; $P = .064$) for disease-free survival.
DISCUSSION
Although it is well known that genetic mutations result in the activation of oncogenes and the inactivation of tumor suppressor genes, increasing evidence has demonstrated that epigenetic alterations, which lead to aberrant DNA methylation, histone modifications, and small noncoding RNA expression, are essential to cancer initiation and progression. EZH2, a histone methyltransferase PcG protein, catalyzes H3-K27 trimethylation, leading to transcriptional inactivation of target genes. EZH2 up-regulation reportedly promoted tumor growth both in vitro and in vivo, and it is present in several cancers in the clinical setting, including melanoma, lymphoma, prostate cancer, and breast cancer. It has been demonstrated that EZH2 is useful as a potential biomarker to distinguish aggressive breast tumors from more benign tumors. In prostate cancer, EZH2 expression has been linked with aberrant H3K27 trimethylation and silencing of potential tumor suppressor genes independent of DNA methylation. The EZH2 expression level also has demonstrated promise as a prognostic indicator in a variety of cancer types, including oral squamous cell carcinoma. Considering the particularity of HNSCC epidemiology, which differs remarkably from other cancer types, as well as the distinct molecular signatures involved in initiation and progression, we evaluated the functional role and expression pattern of EZH2 in HNSCCs. Specifically, we explored the biologic role of EZH2 in HNSCC progression and determined whether EZH2 is a novel immunomarker for patients with HNSCC. Our data demonstrate that EZH2 has a biologic function affecting HNSCC proliferation and invasion in vitro. In addition, aberrant expression of EZH2 in HNSCC was associated with poor differentiation ($P = .020$), histologic signs of severity of HNSCC ($P = .012$), and poorer overall survival ($P = .001$) and disease-free survival ($P = .002$), consistent with the findings described in clinical samples of other cancer types.

A molecular-based model for HNSCC development has been proposed in the literature that includes the deregulation of cell cycle proteins. Cyclin D1 is one of the key cell cycle proteins that frequently is amplified and overexpressed in head and neck tumorigenesis. The overexpression of cyclin D1 has been correlated with...
lymph node metastases in laryngeal squamous cell carcinoma in Chinese patients,\textsuperscript{34} and the up-regulation of cyclin D1 predicted a poor clinical outcome in patients with estrogen receptor-negative breast cancer.\textsuperscript{35} In addition, in our previous study, cyclin D1 ablation in human oral squamous cell carcinoma cells was associated with increased cisplatin chemosensitivity.\textsuperscript{22} Although it has been reported that high EZH2 expression is correlated with the up-regulation of cyclin D1 in gastric cancer, pharmacologic inhibition of EZH2 leads to the down-regulation of cyclin D1 levels in skin cancer.\textsuperscript{24,25} Thus, the correlation between EZH2 expression and cyclin D1 expression in a large cohort of clinical specimens has not been well established. In the current study, we observed that EZH2 overexpression was associated significantly with high cyclin D1 translational levels ($P = .034$) in our population of Chinese patient with HNSCC. However, a significant correlation between EZH2 mRNA and cyclin D1 mRNA was not observed in our Chinese patients with HNSCC, suggesting that EZH2 may affect the post-translational regulation of cyclin D1. It is noteworthy that high EZH2 expression, together with high cyclin D1 levels, was superior in predicting overall survival ($P < .001$) and disease-free survival ($P < .001$) in patients with HNSCC. Further studies need to be performed to support the biological link between EZH2 and cyclin D1 in HNSCC.

In addition, it has been reported that EZH2 is involved in the proliferation of tumor cells and the renewal of stem cells in humans.\textsuperscript{36-40} We observed that HNSCC cells surrounding the cancer nest had elevated levels.

### Table 3. Univariate and Multivariate Cox Proportional Hazards Regression Models for Estimating Overall Survival and Disease-Free Survival

| Characteristics                  | HR    | 95% CI   | P     |
|----------------------------------|-------|----------|-------|
| **Univariate analysis**          |       |          |       |
| Overall survival                 |       |          |       |
| Age (≥60 y vs <60 y)             | 1.091 | 0.672-1.773 | .724  |
| Sex (men vs women)               | 0.777 | 0.443-1.365 | .381  |
| Smoking history (smoker vs nonsmoker) | 0.896 | 0.548-1.465 | .662  |
| Alcohol history (drinker vs nondrinker) | 0.925 | 0.539-1.585 | .776  |
| Tumor grade                      | 1.668 | 1.060-2.625 | .027  |
| TNM stage (III vs IVA)           | 1.538 | 0.909-2.601 | .109  |
| Lymph node metastasis (pN0 vs pN1-pN2) | 2.269 | 1.390-3.703 | .001  |
| EZH2 expression (high vs low)    | 2.226 | 1.353-3.661 | .002  |
| Disease site                     |       |          | .762  |
| Histologic signs of severity (present vs absent) | 2.636 | 1.538-4.520 | <.001 |
| **Disease-free survival**        |       |          |       |
| Overall survival                 |       |          |       |
| Age (≥60 y vs <60 y)             | 0.917 | 0.576-1.460 | .714  |
| Sex (men vs women)               | 0.726 | 0.415-1.267 | .260  |
| Smoking history (smoker vs nonsmoker) | 0.919 | 0.569-1.486 | .732  |
| Alcohol history (drinker vs nondrinker) | 0.85  | 0.499-1.446 | .548  |
| Tumor grade                      | 1.539 | 0.980-2.416 | .061  |
| TNM stage (III vs IVA)           | 1.437 | 0.867-2.381 | .159  |
| Lymph node metastasis (pN0 vs pN1-pN2) | 1.888 | 1.177-3.026 | .008  |
| EZH2 expression (high vs low)    | 2.049 | 1.269-3.308 | .003  |
| Disease site                     |       |          | .763  |
| Histologic signs of severity (present vs absent) | 2.013 | 1.222-3.316 | .006  |
| **Multivariate analysis**        |       |          |       |
| Overall survival                 |       |          |       |
| Tumor grade                      | 1.328 | 0.825-2.139 | .243  |
| EZH2 expression (high vs low)    | 1.863 | 1.090-3.093 | .022  |
| Lymph node metastasis (pN0 vs pN1-pN2) | 1.886 | 1.139-3.122 | .014  |
| Histologic signs of severity (present vs absent) | 2.071 | 1.186-3.617 | .010  |
| **Disease-free survival**        |       |          |       |
| Tumor grade                      | 1.262 | 0.785-2.029 | .337  |
| EZH2 expression (high vs low)    | 1.727 | 1.045-2.856 | .033  |
| Lymph node metastasis (pN0 vs pN1-pN2) | 1.658 | 1.017-2.703 | .043  |
| Histologic signs of severity (present vs absent) | 1.632 | 0.972-2.740 | .064  |

Abbreviations: CI, confidence interval; EZH2, enhancer of zeste homolog 2; HR, hazard ratio; pN, pathologic lymph node status; TNM, tumor-lymph node-metastasis classification.
levels of EZH2 compared with cells that were localized in the center of cancer nest (Fig. 3H). This phenomenon supports evidence indicating that the EZH2 expression level may be linked to the more proliferative potential of HNSCC cells.

It is particularly interesting to note that EZH2 is activated by HPV E7 oncoprotein in cervical carcinogenesis. Although there is diversity in the anatomic site of HNSCC initiation, HPV infection was linked exclusively to the development of oropharyngeal squamous cell carcinoma in approximately 40% of patients. Hence, the correlation between EZH2 expression and high-risk HPV (HPV-hr)/p16 status was evaluated further in patients with oropharyngeal disease from our HNSCC cohort. However, no significant relation between HPV-hr/p16 status and EZH2 expression was observed, probably because of the small number of oropharyngeal squamous cell carcinoma specimens in this study (shown in Table 2). Thus, the clinical and the biologic correlations between HPV-hr/p16 status and EZH2 expression in oropharyngeal squamous cell carcinoma deserve further investigation.

In summary, in this study we used immunohistochemistry to establish that EZH2 expression is strongly associated with cyclin D1 levels in specimens from a large cohort of patients with HNSCC and that EZH2 expression is an independent predictor of clinical outcome in patients with HNSCC. Our findings provide evidence to support the biologic link between EZH2 and cell proliferation and invasion in HNSCC cells. Moreover, our results demonstrate that high EZH2 expression and high cyclin D1 expression together have a superior role in predicting poorer clinical outcome in patients with HNSCC.

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CONFLICT OF INTEREST DISCLOSURES
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