

Aberrant epigenetic regulation in head and neck cancer due to distinct EZH2 overexpression and DNA hypermethylation

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20

Abstract

EZH2 overexpression is associated with tumor proliferation, metastasis, and poor prognosis.

Targeting and inhibiting *EZH2* may be an effective therapeutic strategy for head and neck squamous cell carcinoma (HNSCC). We previously analyzed *EZH2* mRNA expression in a

5 well-characterized dataset of 230 (110 original and 120 validation cohorts) human head and neck cancer samples. This study aimed to investigate the effects of inhibiting *EZH2*, either via RNA interference or via pharmacotherapy, on HNSCC growth. *EZH2* upregulation was significantly correlated with recurrence ($P < 0.001$) and the methylation index of tumor suppressor genes ($P < 0.05$). *DNMT3A* was significantly upregulated upon *EZH2*

10 upregulation ($P = 0.043$). Univariate analysis revealed that *EZH2* upregulation was associated with poor disease-free survival (log-rank test, $P < 0.001$). In multivariate analysis, *EZH2* upregulation was evaluated as a significant independent prognostic factor of disease-free survival (hazard ratio: 2.085, 95% confidence interval: 1.390–3.127; $P < 0.001$). Cells treated with RNA interference and DZNep, an *EZH2* inhibitor, showed the most dramatic changes in
15 expression, accompanied with a reduction in the growth and survival of FaDu cells. These findings suggest that *EZH2* upregulation is correlated with tumor aggressiveness and adverse patient outcomes in HNSCC. Evaluation of *EZH2* expression might help predict the prognosis of HNSCC patients.

20 **Keywords:** *EZH2*, epigenetic regulation, DZNep, tumor-related genes, head and neck cancer

1. Introduction

Head and neck squamous cell cancer (HNSCC) is a heterogeneous disease that potentially involves multiple sites and cellular origins within the head and neck region [1]. Despite aggressive treatments, long-term survival rates are poor and remain at ~50% [2].

Therefore, studies are required to shift their focus from prognostic biomarkers and to the development of predictive biomarkers enabling patient selection for a specific therapy [3,4]. Aberrant gene promoter methylation is a key event in cancer pathogenesis and has gained increasing interest in basic and translational oncology studies because of their reversible nature [5,6]. Therefore, molecular classification of HNSCCs is required to establish prognostic and potentially preventive targets of tumor recurrence in patients.

The enhancer of zeste homolog 2 (*EZH2*) gene, a member of the polycomb repressor complex 2 (PRC2), contributes to the maintenance of cell identity, cell cycle regulation, and oncogenesis [7]. One of the most prominent mechanisms is that many cancer-related genes are silenced by the enhancer of *EZH2*, which can specially promote histone H3 lysine 27 trimethylation (H3K27me3) of target gene promoters [8]. Recently, *EZH2* is reportedly frequently overexpressed in several human epithelial cancer types, including prostate cancer [9], breast cancer [10], gastric cancer [10], non-small-cell lung cancer [11], esophageal cancer [12], melanomas [13], and endometrial carcinomas [14] and is associated with increased tumor cell proliferation and exacerbated outcomes [15,16].

Deazaneplanocin A (DZNep) reportedly downregulates *EZH2* and inhibits H3K27me3 [17]. Although DZNep inhibits proliferation and is a highly promising antimetastatic agent in conditions such as colon cancer, lung cancer, and breast cancer [15,16,18], limited information is available regarding the effects and mechanisms of DZNep in squamous cell carcinoma [19].

Previous studies reported that EZH2 is required for binding of DNA methyltransferases and facilitates CpG methylation of EZH2-target promoters [20]. Repression of the EZH2 target genes results largely from both H3K27me3 and DNA methylation, thereby providing a basis for investigating combinations of both EZH2 and DNA methyltransferase inhibitors in patients with EZH2 overexpression [21,22]. *EZH2* overexpression is correlated with increased promoter methylation across tumor types in The Cancer Genome Atlas (TCGA) [23]. However, a systematic study regarding the epigenetic and transcriptional regulation of *EZH2* genes in HNSCC is still needed.

This study aimed to investigate the biological effects of EZH2 regulation in HNSCC. We assessed the mRNA expression status of *EZH2* genes in HNSCC to evaluate their clinical significance as prognostic biomarkers for recurrence risk and survival. Simultaneous analyses of the *EZH2* expression and the methylation of tumor suppressor genes is important to predict tumorigenesis and the development of future targeted therapy.

2. Results

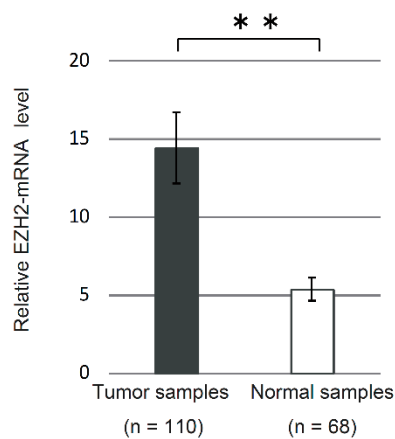
2.1. *EZH2* mRNA expression levels in pairs of HNSCC and normal mucosal tissues

EZH2 mRNA expression levels were determined in 110 HNSCC and 68 adjacent normal mucosal tissues with an independent set (original cohort). *EZH2* mRNA levels were significantly greater in HNSCC tissues than in adjacent normal tissues ($P = 0.003$) (Figure 1A). Furthermore, upon comparing *EZH2* expression in another cohort of 120 pairs of HNSCC and normal tissues (validation cohort), *EZH2* mRNA levels in HNSCC tissues were 3-fold higher than those in paired non-cancerous mucosa ($P = 0.002$) (Figure 1B). *EZH2* mRNA expression exhibited highly discriminative receiver-operator characteristic (ROC)

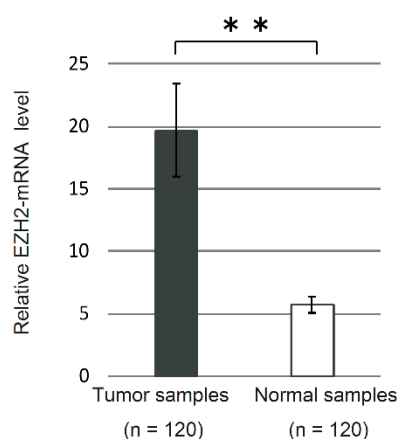
curve profiles, which clearly distinguished HNSCC from normal mucosal tissues (area under the ROC = 0.641). At the cutoff value of 17.81, sensitivity was 33.3%; specificity, 95.1% (Figure 1C).

Figure 1 Kiyoshi Misawa

A Original cohort



B Validation cohort



C

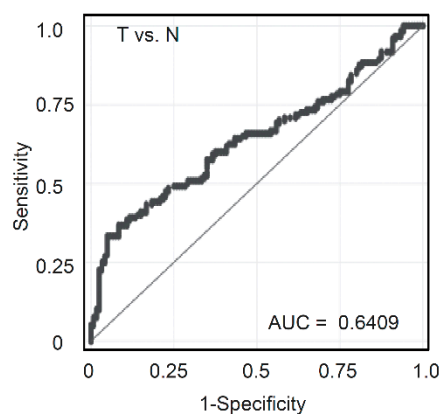


Figure 1. EZH2 mRNA patterns in matched pairs of head and neck squamous cell carcinoma tissue and adjacent normal mucosal tissues

Relative EZH2 mRNA expression levels in (A) the original cohort and (B) the validation cohort were analyzed in clinical samples. Changes between cancerous and normal mucosal tissues were considered significant, as determined by the Student's *t*-test. (C) Area under the ROC curve (AUROC) value for EZH2 was 0.6409. At the cutoff value of 17.81, sensitivity was 33.3%; specificity, 95.1%. ***P* < 0.01.

2.2. Correlation between *EZH2* mRNA expression and clinicopathological assessment

Characteristics and clinicopathologic features of patients are summarized in Table

1. In the original set, *EZH2* mRNA levels were significantly associated with cancer stage ($P = 0.043$) and recurrence ($P = 0.016$). *EZH2* expression in patients in the validation cohort was significantly associated with recurrence ($P = 0.012$). Upon combining the original and validation cohorts, *EZH2* was significantly correlated with recurrence ($P < 0.001$) (Table 1).

Table 1. *EZH2* Gene Expression Status in HNSCC Primary Samples.

Patient and tumor characteristics	Original cohort (n = 110)			Validation cohort (n = 120)			Original and Validation cohorts (n = 230)		
	High (37)	Low (73)	P-value§	High (39)	Low (81)	P-value§	High (76)	Low (154)	P-value§
<i>Age</i>									
Under 70	25	57		26	49		51	106	
70 and older	12	16	1	13	32	0.552	25	48	1
<i>Gender</i>									
Male	31	56		33	70		64	126	
Female	6	17	0.463	6	11	1	12	28	0.715
<i>Alcohol exposure</i>									
Ever	21	39		29	70		50	109	
Never	16	34	0.84	10	11	1	26	45	1
<i>Smoking status</i>									
Smoker	27	45		28	65		55	110	
Non smoker	10	28	0.291	11	16	1	21	44	1
<i>Tumor size</i>									
T1-3	22	56		27	48		49	104	
T4	15	17	1	12	33	0.321	27	50	1
<i>Lympho-node status</i>									
N0	15	36		18	37		33	73	
N+	22	37	0.423	21	44	1	43	81	0.578
<i>Stage</i>									
I, II, III	12	40		20	32		32	72	
IV	25	33	0.043*	19	49	0.243	44	82	0.574
<i>Recurrence events</i>									
Positive	25	31		19	21		44	52	
Negative	12	42	0.016*	20	60	0.012*	32	102	< 0.001*

§ Fisher's exact probability test. * $P < 0.05$

2.3. Prognostic value of *EZH2* gene expression

Kaplan-Meier plots indicated that the expression status of *EZH2* genes was related to disease-free survival (DFS) (Figure 2A-D). In the original cohort, a shorter DFS was also observed when *EZH2* gene upregulation was compared with *EZH2* gene downregulation (log-rank test, $P = 0.002$) (Figure 2A). *EZH2* genes were upregulated in patients in the validation cohort and they had a shorter DFS than those displaying *EZH2* downregulation (log-rank test, $p = 0.024$) (Figure 2B). By combining the original and validation set, DFS rates in patients displaying *EZH2* upregulation was 35.5%, as compared with 56.9% in those displaying *EZH2* downregulation (log-rank test, $P = 0.0001$) (Figure 2C). Moreover, among 152 patients with tumor stage T1, T2, and T3, the DFS rate in those displaying *EZH2* gene upregulation was 39.8%, as compared with 55.6 % in those displaying *EZH2* downregulation (log-rank test, $P = 0.009$) (Figure 2D). Furthermore, the association between methylation and risk of recurrence was estimated via multivariate analysis by using a Cox proportional hazards model adjusted for age, sex, alcohol exposure, smoking status, and clinical stage. In patients displaying *EZH2* upregulation (76/230, 33.0%), the adjusted odds ratio (OR) for recurrence was 2.085 (95% confidence interval [CI]: 1.390–3.127, $P < 0.001$) (Table 2).

Figure 2 Kiyoshi Misawa

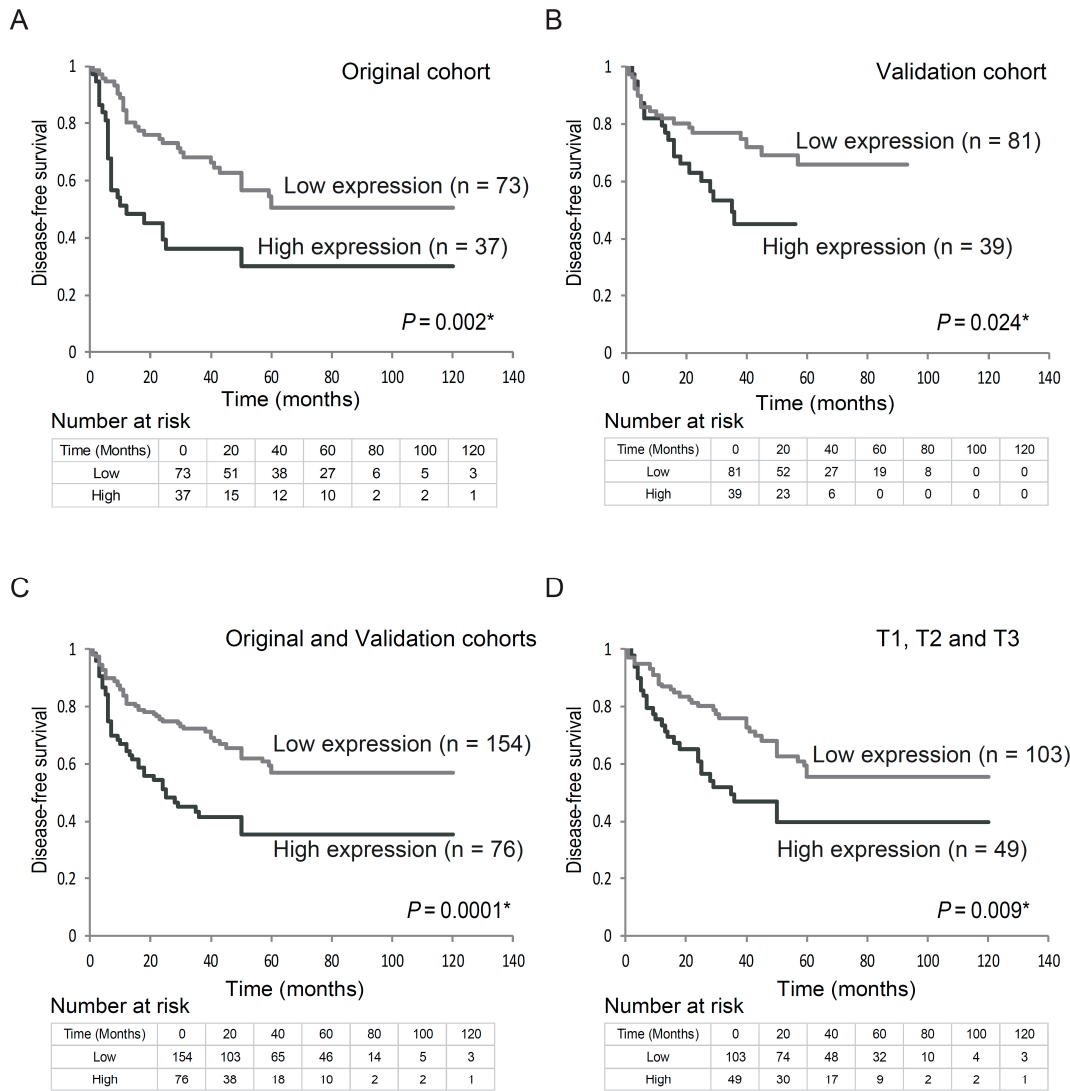


Figure 2. Kaplan-Meier survival curves for patients with head and neck squamous cell carcinoma (HNSCC) based on EZH2 gene expression status

Kaplan–Meier survival curves based on EZH2 gene expression status in patients with

5 HNSCC. Disease-free survival based on (A) the original cohort, (B) the validation cohort, (C) the original and the validation cohort, (D) and the original and the validation cohort in tumor stage 1, 2, and 3 patients. Gray and black lines indicate patients with tumors displaying EZH2 downregulation and upregulation, respectively.

Table 2. Multivariate analysis of factors affecting survival using Cox proportional hazards model in 230 HNSCC patients.

Variables	Disease-free survival	
	HR (95% CI)	P
<i>Age</i>		
70 and older vs. < 70	1.326 (0.860-2.044)	0.202
<i>Sex</i>		
Male vs. Female	1.065 (0.615-1.846)	0.821
<i>Alcohol exposure</i>		
Ever vs. Never	0.516 (0.308-0.865)	0.012*
<i>Smoking status</i>		
Smoker vs. Non smoker	1.988 (1.127-3.505)	0.018*
<i>Stage</i>		
I, II, III vs. IV	1.672 (1.088-2.568)	0.019*
<i>EZH2 expression</i>		
High vs. Low	2.085 (1.390-3.127)	< 0.001*

HR: hazard ratio

95% CI: 95% confidence interval

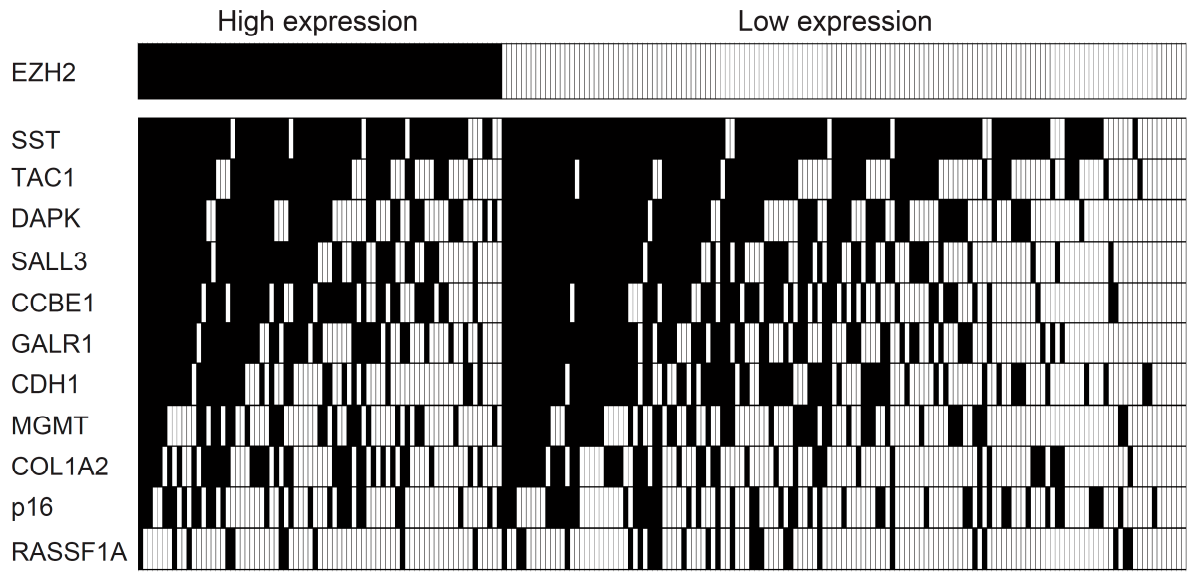
* P < 0.05

2.4. Correlation between methylation levels of 11 tumor suppressor genes and *EZH2* expression

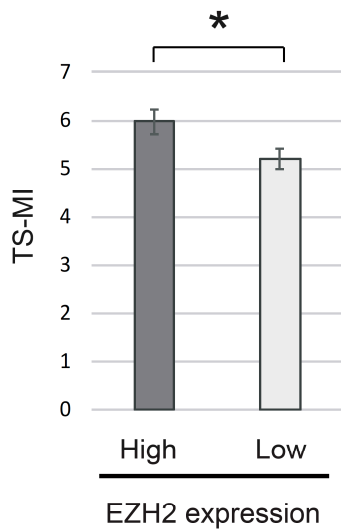
The 11 tumor suppressor genes were defined as the number of methylated genes in each sample (Figure 3A, Supplementary Table S3). Mean differences in the methylation index of the 11 tumor suppressor genes (TS-MI) determined on the basis of *EZH2* gene expression are illustrated in Figure 3B. In particular, the TS-MI was significantly higher in patients displaying *EZH2* upregulation (5.97 ± 0.25) than in those with displaying *EZH2* downregulation (5.21 ± 0.22 , P = 0.032) (Figure 3B). We examined DNA methyltransferase 3 alpha (*DNMT3A*) and 3 beta (*DNMT3B*) genes mRNA levels via qRT-PCR in HNSCC specimens. *DNMT3A* was significantly upregulated in *EZH2* higher expression groups (P = 0.043) (Figure 3C). *DNMT3B* expression was not associated with *EZH2* expression (P = 0.628) (Figure 3D).

Figure 3 Kiyoshi Misawa

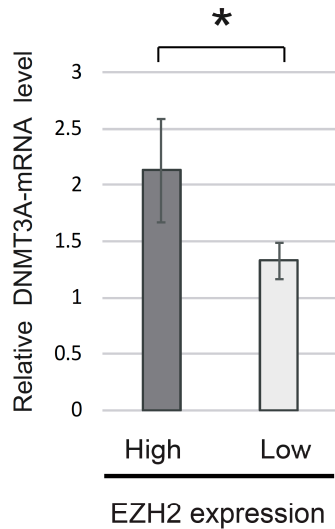
A



B



C



D

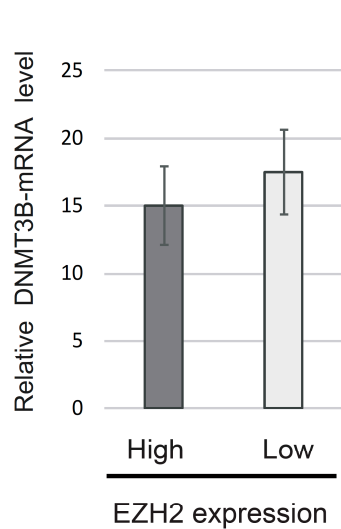


Figure 3. Comparison of methylation rates in 11 tumor suppressor genes along with EZH2 expression in 230 primary head and neck squamous cell carcinoma (HNSCC) tissues

(A) Distribution of EZH2 gene expression status and 11 tumor suppressor genes promoter

methylation. Shaded boxes indicate the presence of methylation, and open boxes indicate the

absence of methylation. (B) Correlation between the methylation index of tumor suppressor genes (TS-MI) and *EZH2* gene expression status in HNSCC patients ($P = 0.032$). The mean TS-MI for the different groups were compared using Student's *t*-test. (C) *DNMT3A* mRNA was significantly upregulated in groups displaying *EZH2* upregulation ($P = 0.032$). (D)

5 *DNMT3B* expression was not associated with *EZH2* expression status ($P = 0.628$). * $P < 0.05$.

2.5. siRNA-mediated *EZH2* knockdown and DZNep administration

We performed in vitro experiments to investigate the effect of siRNA-mediated knockdown of *EZH2* protein, using the *EZH2*-expressing HNSCC cell line FaDu. *EZH2* siRNA significantly downregulated *EZH2* protein and mRNA, as evident from Western blot
10 analysis ($P < 0.05$) (Figure 4A) and qRT-PCR analysis ($P < 0.05$) (Figure 4B), respectively. In addition, FaDu cells with *EZH2* downregulation showed a significantly reduced colony formation ability ($P < 0.05$) (Figure 4C). As expected, DZNep treatment significantly downregulated *EZH2* mRNA ($P < 0.05$) (Figure 4D). After treating cells with DZNep for 5 and 7 d, DZNep significantly inhibited cellular proliferation temporally, as evident from the
15 growth curve ($P < 0.05$ and $P < 0.01$, respectively) (Figure 4E).

Figure 4 Kiyoshi Misawa

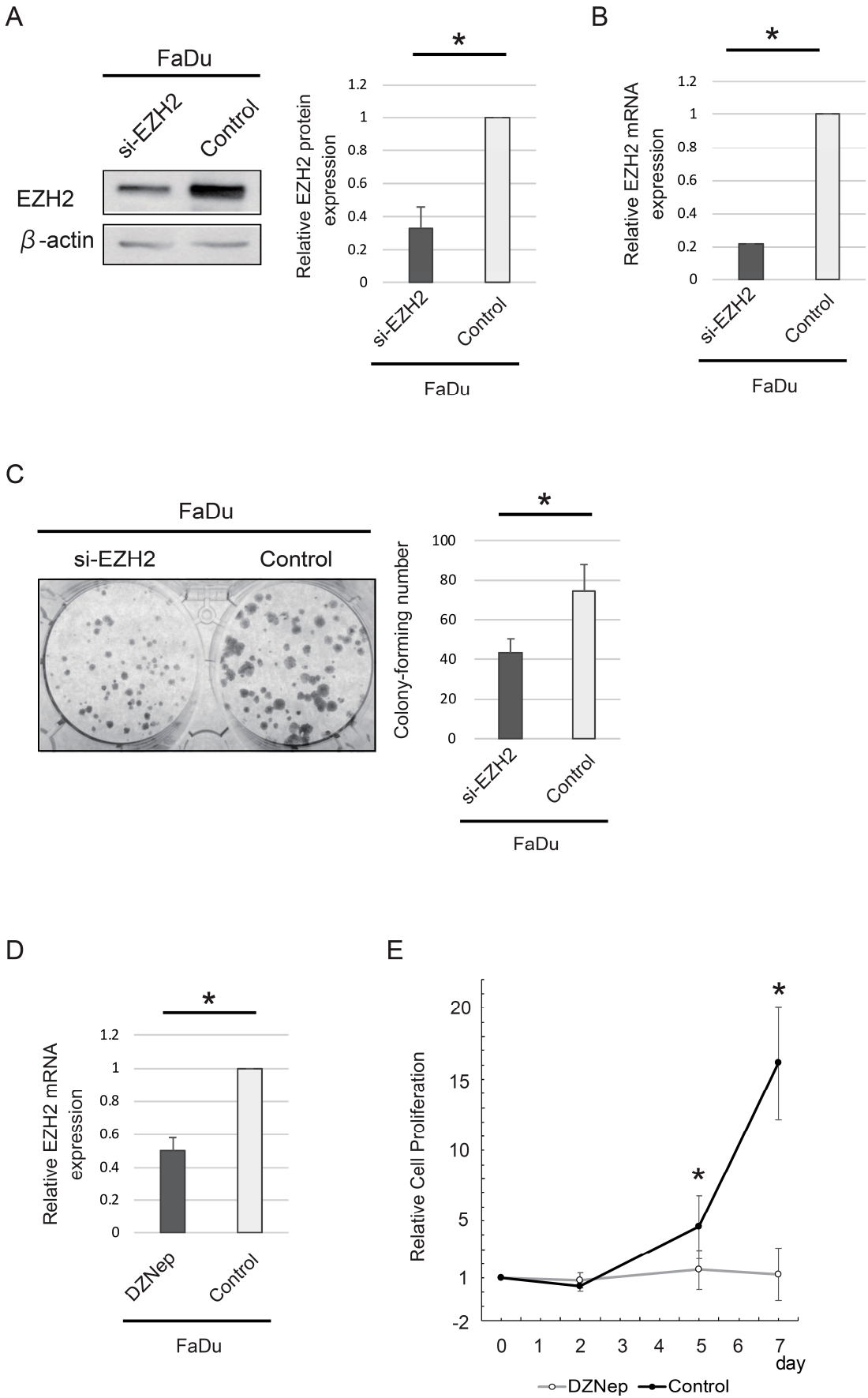


Figure 4. siRNA- and DZNep-mediated EZH2 knockdown inhibited the proliferation of FaDu cells

(A) Western blot analysis of EZH2 protein expression after siRNA transfection. (B) The chart illustrates the quantification of EZH2 mRNA expression levels via quantitative reverse transcription polymerase chain reaction (qRT-PCR) after siRNA-mediated knockdown of EZH2 mRNA. (C) Colony formation upon transfection with EZH2-targeting siRNA or control. (D) EZH2 mRNA expression was analyzed via qRT-PCR from FaDu cells treated with DZNep for 48 h. (E) Effect of DZNep (10 μ M) on the growth curve. Experiments were performed in duplicate and repeated thrice. The mean \pm standard deviation value for each treatment is graphed here. * $P < 0.05$. ** $P < 0.01$.

2.6. External validation of results from TCGA

Data from 43 non-tumorous tissues and 497 HNSCC tissues were analyzed. The EZH2 gene expression status was significantly higher in the HNSCC samples than in the normal samples ($P < 0.001$) (Supplementary Figure S1A). Spearman analysis revealed that EZH2 expression was positively correlated with DNMT3A and DNMT3B expression ($R^2 = 0.0734$, $P < 0.001$ and $R^2 = 0.02$, $P = 0.002$, respectively) (Supplementary Figure S1B, 1C).

3. Discussion

This study reported that EZH2 was upregulated in HNSCC cells in comparison with normal cells, being associated with a higher recurrence risk. Based on these findings, our study is the first, to our knowledge, to report a quantitative correlation between EZH2 overexpression and DNA hypermethylation in HNSCC. Herein, we identified that DZNep, a small-molecule EZH2 inhibitor, as a putative therapeutic target of HNSCC. These results suggest that EZH2 inhibition is a promising therapeutic avenue for a substantial fraction of

HNSCC patients. Clarification of the epigenetic regulation of *EZH2* gene may provide insights into the underlying mechanisms of tumorigenesis and the risk of disease recurrence in HNSCC.

EZH2 expression is reportedly associated with aggressive prostate cancer [24],

5 esophageal cancer [12], and breast cancer [24], whereas its clinical importance in HNSCC is

yet unknown. *EZH2* has gained interest as a marker of aggressive subgroups in several

cancers [25]. DNZep is an S-adenosylhomocysteine (SAH) hydrolase inhibitor causing

intracellular SAH levels to increase [26]. DNZep treatment induces chondrosarcoma death

via apoptosis, while it decreases their migration ability [17]. DNZep can directly induce cell

10 senescence, along with apoptosis, in human colon cancer [18]. The PRC2 inhibitory effects of

DNZep are important for differentiation-inducing and anticancer activities observed in

HNSCC [27].

EZH2 constitutes the catalytic subunit of PRC2 and catalyzes H3K27me3 and mediates the silencing of target genes via local chromatin reorganization [28]. *EZH2*

15 reportedly promotes cell proliferation, migration, and invasion in different in vitro cancer cell

models [29]. Viré E et al. reported that *EZH2* regulates CpG methylation through direct

physical contact with DNA methyltransferases [20]. *EZH2* overexpression was correlated

with increased promoter methylation across numerous tumor types in prostate cancer, small-

cell lung cancer, and clear cell sarcoma of the kidney [22,23,30]. However, a systematic

20 study of *EZH2* expression and DNA promoter methylation in most human cancers is still

needed. To our knowledge, this study is the first to implicate *EZH2* expression and DNA

promoter methylation in the genesis of HNSCC.

Thus, *EZH2* may play a specific role depending on the cell type and different sites of tumorigenesis. A study of this type involving human specimens and utilizing high-throughput profiling platforms may be susceptible to measurement bias from various sources. Our results suggest, for the first time, that increased *EZH2* expression is correlated with tumor progression and may facilitate the accumulation of aberrantly methylated tumor suppressor genes in HNSCC. Moreover, transcriptional activation of *EZH2* was associated with aberrant methylation of other tumor-related genes and *DNMT3A* upregulation in HNSCC. The present results suggest that the targeted inhibition of *EZH2* catalytic activity may constitute a promising therapeutic intervention; however, additional prospective studies are required to prevent broad side effects.

4. Materials and methods

4.1. Tumor samples

Two independent cohorts comprised 110 and 120 (original and validation cohorts) fresh frozen samples of primary HNSCC obtained from patients from the Hamamatsu University Hospital. Written informed consent was obtained from all patients in accordance with the tenets of the Declaration of Helsinki and the study protocol was approved by the Institutional Review Board of the Hamamatsu University School of Medicine. Original sets of HNSCC tissues were collected from January 1995 to December 2004. For the validation set, we obtained tumor tissues and adjacent normal tissues from October 2005 to September 2016. Clinical information, including age, sex, tumor site, smoking habit, alcohol consumption, tumor size, lymph node status, and stage grouping were obtained from the patients' clinical records. Supplemental Table S1 summarizes the demographic characteristics of the study population.

4.2. Cell culture

The SCC cell line FaDu was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 10% fetal bovine serum (ThermoFisher Scientific Inc., Waltham, MA, USA) and 1% penicillin/streptomycin (FUJIFILM Wako Pure Chemical Corporation) in a humidified atmosphere containing 5% CO₂ at 37°C.

4.3. RNA extraction and quantitative reverse transcription PCR (qRT-PCR)

Total RNA was isolated using an RNeasy Plus Mini Kit (Qiagen, Hilden, Germany), and cDNA was synthesized using a ReverTra Ace qPCR RT Kit (Toyobo, Tokyo, Japan). *EZH2*, *DNMT3A*, *DNMT3B*, and *GAPDH* mRNA expression levels were measured via qRT-PCR using SYBR Premix Ex Taq (Takara Bio Inc, Tokyo, Japan), the Takara Thermal Cycler Dice Real Time System TP8000 (Takara Bio Inc) and the primer sets presented in Supplemental Table S2. Target gene expression levels were normalized to those of *GAPDH* using the $2^{-\Delta\Delta C_t}$ method.

4.4. EZH2 siRNA transfection, colony forming assay, and cell proliferation assay

EZH2 siRNA (EZH2HSS176653, Invitrogen, Thermo Fisher Scientific, MA, USA) was diluted in Opti-MEM I Reduced Serum Medium (Invitrogen) and Lipofectamine RNAiMAX Transfection Reagent (Invitrogen). siRNA complexes were transfected into cells at a final concentration of 50 nM for RNA and protein assays, 20 nM for the colony forming assay, and 0 nM in the control cells, in accordance with the manufacturer's instructions. The EZH2 functional inhibitor, DZNep (Sigma-Aldrich, St. Louis, MO, USA), was added to FaDu medium at 10 μ M for the cell proliferation assay. For the colony forming assay, cells

(0.05×10^4 cells) were placed into each well of 6-well plates and allowed to grow for 14 d, following which plates were fixed and stained with 40% ethanol-1% crystal violet and enumerated in a field of >0.1 mm diameter. For the cell proliferation assay, cells (0.2×10^4 cells) were placed into each well in 12-well plates enumerated and at 3 time points (day 2, day 5, and day 7).

4.5. Western blot analysis

Cells were lysed using RIPA Buffer (FUJIFILM Wako Pure Chemical Corporation) containing Protease Inhibitor Cocktail (Promega, Madison, WI, USA). The supernatant was harvested, and protein content was measured using the Protein Assay Rapid Kit WAKO II (FUJIFILM Wako Pure Chemical Corporation). The proteins were separated via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% TGX FastCast Acrylamide Kit gels (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and electroblotted onto Immobilon-P polyvinylidene difluoride membranes (Merck KGaA, Darmstadt, Germany). The membranes were incubated overnight with anti-EZH2 antibody (Cat. No. 612667, BD, NJ, USA) and anti- β -actin antibody (Cat. No. A2228, Merck KGaA) at 4°C , followed by incubation with biotin-conjugated secondary anti-mouse antibody (Nichirei Biosciences INC., Tokyo, Japan) followed by the horseradish peroxidase-conjugated avidin-biotin reaction (Nichirei Biosciences INC.). Immunoreactive bands were visualized using an enhanced chemiluminescence substrate (GE Healthcare UK Ltd, Buckinghamshire, UK). Western blot images were obtained using a ChemiDoc Touch imaging system (Bio-Rad Laboratories, Inc.) and analyzed using Image Lab 5.2 (Bio-Rad Laboratories, Inc.).

4.6. DNA extraction, bisulfite treatment, and quantitative methylation-specific PCR (QMSP) analysis

Extraction and bisulfite conversion of genomic DNA from 216 primary HNSCC samples were performed using the MethyLEasy Xceed Rapid DNA Bisulfite Modification Kit (Takara Bio Inc) in accordance with the manufacturer's instructions. QMSP was performed as described previously [31]. The QMSP primer sequences for *CCBE1*, *CDH1*, *COL1A2*,
 5 *DAPK*, *GALR1*, *MGMT*, *p16*, *RASSF1A*, *SALL3*, *SST*, and *TAC1* are provided in Supplementary Table S2. Overall methylation levels in individual samples were determined by calculating the methylation value. TS-MI was defined as the ratio of the number of methylated genes to the number of tested genes in each sample [32].

4.7. Collection of publicly available data from TCGA

10 Gene expression data were obtained from the TCGA data portal (<https://tcga-data.nci.nih.gov/tcga/>) and MethHC (<http://methhc.mbc.nctu.edu.tw/php/index.php>) in July 2018 [33]. Expression data were obtained as processed RNA-seq data in the form of RNA-seq via Expectation Maximization.

4.8. Data analysis and statistics

15 The *EZH2* expression status results and patient characteristics were compared using the Student's *t*-test. Receiver operating characteristic (ROC) curve analysis was performed using for 120 HNSCC and 120 adjacent normal mucosal samples and the Stata/SE 13.0 system (Stata Corporation, College Station, TX, USA). Area under the ROC curve analysis indicated the optimal sensitivity and specificity cut-off levels to differentiate
 20 between gene expression levels in HNSCC and normal tissue.

DFS was measured from the date of the initial treatment to the date of diagnosis of locoregional recurrence or distant metastasis. The Kaplan-Meier test was used to calculate survival probabilities, and the log-rank test was used to compare survival rates. The

prognostic value of *EZH2* expression status was assessed by performing multivariate Cox proportional hazards analysis adjusted for age (≥ 70 versus < 70 years), sex, alcohol intake, smoking status, and tumor stage (I, II, and III versus IV). Differences with $P < 0.05$ were considered significant. All statistical analyses were performed using the StatMate IV software (ATMS Co. Ltd., Tokyo, Japan).

5. Conclusions

In conclusion, the present results suggest that *EZH2* is aberrantly expressed in HNSCC patients. CpG island hypermethylation is an early event in tumorigenesis, supporting the hypothesis that *EZH2* may play an important role in tumorigenesis in HNSCC and may serve as an important biomarker. We demonstrated for the first time, to our knowledge, that *EZH2* mRNA is downregulated in HNSCC owing to DNA methylation; this may be a critical event in HNSCC progression, which is associated with decreased survival.

Supplementary Materials:**Figure S1. Data regarding DNA methylation obtained from The Cancer Genome Atlas**

(TCGA). Data regarding *EZH2*, *DNMT3A*, and *DNMT3B* mRNA expression in head and neck squamous cell carcinoma were obtained from TCGA (<https://tcga-data.nci.nih.gov/tcga/>)

5 and MethHC (<http://methhc.mbc.nctu.edu.tw/php/index.php>).

Table S1. Baseline Characteristics of the 230 Patients.**Table S2. Q-RT-PCR and Q-MSP primer list****Table S3. EZH2 expression status with the methylation of other eleven genes.**

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Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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