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EZH2 is associated with poor prognosis in head-and-neck squamous cell carcinoma via regulating the epithelial-to-mesenchymal transition and chemosensitivity

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SUMMARY

Objectives: Increasing evidence suggests that epigenetic regulation is responsible for tumor initiation and progression in head and neck squamous cell carcinoma (HNSCC). Although the polycomb group protein enhancer zeste homolog 2 (EZH2) is upregulated and a key epigenetic modifier implicated in various cancers, its molecular mechanism in HNSCC remains unknown. Herein, we investigated the role of EZH2 in HNSCC progression and its clinical implication as an HNSCC risk predictor.

Materials and method: A retrospective analysis was performed on 90 HNSCC patients who had curative surgery between 1999 and 2011. Patients with high and low EZH2 expression were compared by the various clinicopathological factors. Survival rates were estimated by the Kaplan–Meier method and log-rank test was used to determine significance. For functional *in vitro* analysis, migration/invasion assay and Western blotting were performed after EZH2 knockdown using siRNA. In addition, cell proliferation was measured to clarify the role of EZH2 on cisplatin chemotherapy.

Results: In patients with HNSCC, high EZH2 expression was correlated with advanced T stage and poor survival outcome. RNAi analysis revealed that EZH2 silencing increased E-cadherin expression while decreasing that of N-cadherin and Vimentin without altering Snail/Slug signaling, which led to decreased cell migration/invasion. EZH2 is also associated with tumor aggressiveness via regulating the epithelial-to-mesenchymal transition. Furthermore, we show that high EZH2 expression decreases sensitivity to cisplatin-based chemotherapy.

Conclusion: Our results indicate that EZH2 may not be only a predictive and prognostic biomarker but also a potential personalized therapeutic target for the treatment of HNSCC.

Introduction

Head-and-neck squamous cell carcinoma (HNSCC) is the sixth most common cancer in the world (1.6 million diagnosed as new cases; 333,000 deaths recorded by HNSCC annually) and occurs as a heterogeneous tumor with an aggressive phenotype [1]. However, despite the advances in biology and medicine over the past several decades, the prognosis of HNSCC remains dismal under current treatment strategies, and more than 50% of patients die of HNSCC and related conditions within five years [2]. This is most likely due to a lack of understanding the molecular basis of HNSCC, as well as potent biomarkers to predict HNSCC progression

Abbreviations: AJCC, American Joint Committee on Cancer; DAB, diaminobenzidine; DFS, disease free survival; EMT, epithelial-to-mesenchymal transition; EZH2, enhancer of zeste homolog 2; FBS, fetal bovine serum; H3-K27, histone H3 lysine 27; HNSCC, head and neck squamous cell carcinoma; HPV, human papilloma virus; IHC, immunohistochemical; OS, overall survival; PRC2, polycomb repressive complex 2; SDS, sodium dodecyl sulfate; TMA, tissue microarray.

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or therapeutic resistance [3]. However, the development of HNSCC is an evolutionary process characterized by multistep carcinogenic processes in which activation of oncogenes and inactivation of tumor suppressor genes – including p53, epidermal growth factor receptor, Ras, MYC, survivin, cyclin D1, and cyclin-dependent kinase inhibitor – are caused by genetic alterations and epigenetic modifications that lead to the aberrant proliferation and aggressiveness of tumor cells [4].

Enhancer of zeste homolog 2 (EZH2) is the catalytic subunit of polycomb repressive complex 2 (PRC2), a highly conserved histone methyltransferase that methylates histone H3 lysine 27 (H3-K27) [5]. H3-K27 trimethylation is commonly associated with DNA methylation and the silencing of genes responsible for differentiation in humans [5]. It has been demonstrated that EZH2 is involved in methylation and silencing of a subset of genes implicated in cell differentiation, suggesting that it plays a key role in cell differentiation and maintenance of adult stem cell populations [6]. Moreover, it has been reported that EZH2 is frequently overexpressed in several human malignancies, indicating that EZH2 plays a critical role in carcinogenesis, and is implicated in tumor cell proliferation and invasion [7]. Furthermore, recent studies suggest that EZH2 is related to drug resistance in several types of cancer [8–10]. However, the association between EZH2 and chemotherapy resistance to HNSCC has not yet been determined.

In the present study, we aimed to investigate the role of EZH2 in HNSCC pathophysiology by applying an *in vitro* functional assay combined with expression analysis using a tissue microarray (TMA) and a retrospective analysis of HNSCC patient cohorts. Our ultimate goal was to address whether EZH2 expression status in primary HNSCC could serve as a prognostic marker for patients and predict the response to platinum-based chemotherapy for HNSCC.

Materials and methods

HNSCC patients

We retrospectively reviewed the medical charts of a study cohort consisting of 90 HNSCC patients who had undergone curative surgery (primary resection and appropriate cervical LN dissection according to disease stage) at the Department of Otolaryngology-Head and Neck Surgery in Chungnam National University Hospital from April 1999 to December 2011. This study was approved by the Institutional Review Board of Chungnam National University College of Medicine (Jung-gu Daejeon, Korea), and the requirement to obtain informed consent was waived. Clinicopathological characteristics of the patients are summarized in Table 1. Among these patients, 30 (33.3%) had oral cavity cancer, 16 (17.8%) had oropharyngeal cancer, 10 (11.1%) had hypopharynx cancer, and 34 (37.8%) had larynx cancer. Tumor size and stage were classified according to the TNM system by the American Joint Committee on Cancer (AJCC), and tumor differentiation was classified according to the World Health Organization (WHO) classification of histologic differentiation. The mean follow-up duration was 32.8 months (range, 2–122 months).

Tissue microarray (TMA) construction

Formalin-fixed paraffin-embedded tumor blocks were collected and TMAs generated as described previously [11]. Briefly, sections were cut from each donor block and stained with hematoxylin and eosin (H&E) to identify the tumor area. A small tissue core (two cylinders per patient) with a diameter of 2 mm was taken from the donor block using a tissue chip microarray (Beecher Instruments, Silver Spring, MD) and transferred to a recipient paraffin block. Histologic sections (5 μm thickness) were cut from the recipient paraffin block using standard techniques.

Table 1
Clinicopathological characteristics of 90 HNSCC patients.

Variable	Total	Primary site (number)			
		Oral cavity (n = 30)	Oropharynx (n = 16)	Hypopharynx (n = 10)	Larynx (n = 34)
Age range (years)	27–87	27–87	42–71	46–73	49–80
Mean (years)	68	67	68	57	72
Gender					
Male	82	24	14	10	34
Female	8	6	2	0	0
Tumor differentiation					
Well	55	20	8	8	19
Moderate	23	9	4	1	9
Poor	12	1	4	1	6
Tumor size					
I + II	46	24	12	3	7
III + IV	44	6	4	7	27
LN involvement					
No	42	19	4	4	15
Yes	48	11	12	6	19
AJCC stage					
I + II	28	18	5	3	5
III + IV	62	12	11	7	29
HPV status					
Positive		7	7	2	7
Negative		23	9	8	27
Recurrence					
No	53	15	10	7	21
Yes	37	15	6	3	13
PreOP RTx					
No	77	26	13	9	29
Yes	13	4	3	1	5

LN, lymph node; AJCC, American Joint Committee on Cancer; PreOP RTx, preoperative radiotherapy.

Immunohistochemistry and scoring system

Immunohistochemical (IHC) staining using an anti-EZH2 antibody (Cell Signaling Technology Inc., Danvers, MA, 1:50) was performed using a 3,3′-diaminobenzidine (DAB) peroxidase substrate kit according to the manufacturer's instructions (Sigma–Aldrich, St. Louis, MO). At least two experienced pathologists without access to clinical patient information analyzed slides under light microscopy (400× magnification). Results were classified according to two parameters from a modified method described previously [12]: EZH2 staining extent (score: 0, no staining; 1, <35% positive cells; 2, 35–75%; 3, >75%) and staining intensity (score: 0, no staining; 1, weak; 2, moderate; 3, strong). By multiplying the staining extent by the intensity, we obtained the IHC staining grade (range, 0–9). For semi-quantitative analysis, grade 0 was considered no staining, grades 1 and 2 were considered weak staining (+1), grades 3 and 4 were considered moderate staining (+2), and grades 6 and 9 were considered strong staining (+3). Finally, for statistical comparison, specimens with no staining and (+1) were included in the low EZH2 group, and those with (+2) and (+3) were included in the high EZH2 group.

Detection of human papilloma virus (HPV) DNA by in situ hybridization

DNA *in situ* hybridization was performed on tumor formalin-fixed paraffin-embedded sections from each case using the catalyzed reporter deposition system for the detection of HPV16 DNA as previously described [13].