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Role of EZH2 in oral squamous cell carcinoma carcinogenesis

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ABSTRACT

Oral squamous cell carcinoma (OSCC) is a common human malignancy with high incidence rate and poor prognosis. Although the polycomb group protein enhancer of zeste homolog 2 (EZH2) plays a crucial role in cell proliferation and differentiation during the occurrence and development progress of several kinds of malignant tumors, the impact of EZH2 on the development and progression of OSCC is unclear. In this study, we demonstrate that EZH2 is overexpressed in OSCC cells and clinical tissue. With *in vitro* RNAi analysis, we generated stable *EZH2* knocking down cell lines from two OSCC cell lines, with two sh-RNAs targeting to *EZH2*, respectively. We found that knocking down of *EZH2* could decrease the proliferation ability and induce apoptosis of OSCC cells. Moreover, we demonstrated that of EZH2 inhibition decreased the migration and metastasis of OSCC cells. In conclusion, the results of the current study demonstrated an association between EZH2 expression and OSCC cell development. We recommend that *EZH2* acts as an oncogene and plays an important role in OSCC carcinogenesis.

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer globally. There are 1.6 million new cases diagnoses and 333000 deaths caused by HNSCC annually, with half are localized in the oral cavity (oral squamous cell carcinoma, OSCC) (Jemal et al., 2009). Oral squamous cell carcinoma has been an important component of the worldwide burden of cancer, with the 5-year survival rate approximately 50%, which is poorer than breast cancer or melanoma (Jemal et al., 2011). The occurrence and development of OSCC are complex involving many genes and pathways. The mechanism of OSCC development remains unclear.

Several proto-oncogenes have been reported to be involved in OSCC, including *RAS*, epithelial growth factor receptor (*EGFR*), *MYC*, survivin, and Cyclin D1 (Mishra and Das, 2009; Murugan et al., 2012; Pai, 2009; Preuss et al., 2008; Sarkis et al., 2010). Overexpression or mutation of these genes is associated with abnormal cell proliferation and tumor aggressiveness (Choi and Myers, 2008; Scully, 2011). Revealing the molecular mechanisms underlying the pathogenesis and progression of oral squamous cell carcinoma may lead to the development of new and effective strategies for diagnosis, prognostication, early detection, and targeted therapy.

The polycomb group protein enhancer of zeste homolog 2 (EZH2), a specific histone 3 lysine 27 (H3K27) methyltransferase, plays a critical role in epigenetic gene silencing and chromatin remodeling. It has a master regulatory function in cell proliferation and differentiation (Hock, 2012; Wu et al., 2011). Overexpression of EZH2 has been related to repression of tumor suppressor genes and derepression of genes involved in metastasis, and has been shown to exert oncogenic effects on various types of tumors, including human breast cancer, prostate cancer, gastric cancer, hepatic carcinoma, bladder cancer, kidney cancer, and ovarian cancer (Alford et al., 2011; Chang et al., 2011; He et al., 2012, 2010; Karanikolas et al., 2009; Kim et al., 2013; Raman, 2005; Sasaki et al., 2008; Wagener et al., 2010). EZH2 promotes the progression of regulating cell cycle and apoptosis in cholangiocarcinoma cells and down-regulation in breast cancer reduces *in vivo* tumor growth (Gonzalez et al., 2009; Nakagawa et al., 2013). Previous research has shown that overexpression of EZH2 was related to malignant potential and adverse outcomes in OSCCs, while a functional study of the role of EZH2 in the development and progression of OSCC has not yet been investigated (Kidani et al., 2009).

The purpose of this study was to investigate the biological function of EZH2 in OSCC. We detected EZH2 in OSCC cells to elucidate the

Abbreviations: OSCC, oral squamous cell carcinoma; HNSCC, head and neck squamous cell carcinoma; EZH2, enhancer of zeste homolog 2; *EGFR*, epithelial growth factor receptor; H3K27, a specific histone 3 lysine 27; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PVDF, polyvinylidene difluoride; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

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function and the influence of EZH2 on cell proliferation, apoptosis, metastasis and invasion of OSCC cells.

2. Materials and methods

2.1. Cell culture and tissue specimens

The human OSCC cell lines Tca8113, Tb, Ts, and the human adenoid cystic carcinoma cell lines ACC-M and ACC-2 were cultured in RPMI-1640 medium. The human OSCC cell lines CAL27 and SCC-4, and human skin keratinocyte cell line HaCaT were grown in Dulbecco's modified Eagle's medium (DMEM). These cells are all supplemented with 10% fetal bovine serum (FBS) (PAA Laboratories GmbH, Pasching, Australia), at 37 °C in a humidified 5% CO_2 atmosphere. Tissues of OSCC and the normal specimens were obtained from surgical specimens immediately after resection from patients. The samples were flash frozen in liquid nitrogen and stored at −80 °C until use. All specimens were randomly selected at the Second Affiliated Hospital of Harbin Medical University, China.

2.2. RNA isolation and quantitative reverse transcription PCR (qRT-PCR)

For *EZH2* gene expression analysis, total RNA was isolated using TRIzol reagent (Invitrogen Inc., Carlsbad, USA). The cDNA synthesis was generated from a First-Strand cDNA Synthesis Kit (Promega, Madison, WI, USA), according to the instructions of the supplier. qRT-PCR was performed on LightCycler 480 (Roche Diagnostics Ltd, Rotkreuz, Switzerland). Assays were performed in 20 μl reaction mixtures, using a LightCycler 480 SYBR Green I Master Kit, following the manufacturer's protocol. The primers used to amplify *EZH2* were: (F) 5′-CAT GTG CAG CTT TCT GTT CAA-3′ and (R) 5′-AGT CTG GAT GGC TCT CTT GG-3′. The primers used to amplify the β-actin control gene were: (F) 5′-AAA TCT GGC ACC ACA CCT TC-3′ and (R) 5′-GGG GTG TTG AAG GTC TCA AA-3′. All measurements were done in triplicate. The threshold cycle value for each product was determined and normalized to that of the internal control, β-actin. qRT-PCR results were analyzed using LightCycler 480 version 1.5.0 software.

2.3. Immunoblotting analysis

Cells were harvested in logarithmic phase and were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, 1 mM EDTA, 50 mM Tris–HCl, pH 7.4). Proteins were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membranes were incubated with the anti-EZH2 antibody (Cell Signaling Technology Inc., Danvers, MA, USA) overnight at 4 °C, and then incubated with fluorescent-labeled secondary antibody (Zhongshan Bio-Tech Co., Beijing, China) for 1 h at room temperature. GAPDH was detected as control with the anti-GAPDH antibody (KangChen Biotech., Shanghai, China). The membranes were then scanned using the Odyssey infrared imaging system (LICOR, Lincoln, NE, USA).

2.4. Generation of stable oral cancer cell lines knocking down EZH2

Oligonucleotides encoding a siRNA specific for *EZH2* were subcloned into pLKO.1-TRC vector (Addgene, Cambridge, MA, USA). The following target sequences of *EZH2* have been selected: 5′-AAACAGCTGCCTTA GCTTCA-3′ (sh-*EZH2*-1), and 5′-GCTAGGTTAATTGGGACCAAA-3′ (sh-*EZH2*-2). sh-Control is: 5′-CTGGCATCGGTGTGGATGA-3′. The authenticity of these plasmids was confirmed by sequencing. Tca8113 cells and CAL27 cells were transfected with sh-*EZH2* or sh-control vector by Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA), in accordance with the manufacturer's instructions. Stable cell lines were established after 2 weeks of G418 (200 μg/ml to Tca8113 cells, and 300 μg/ml to CAL27 cells) selection, and the expression of EZH2 was confirmed by immunoblotting analysis.

2.5. Cell proliferation assay and colony formation assay

Two thousand Tca8113 cells and CAL27 cells were seeded in 96-well plates. Then cells were performed with MTS using CellTiter 96® AQueous One Solution Cell Proliferation Assay Kit (Promega Corporation, Madison, WI, USA). The OD value of each well was read for every 24 h for continuously 5 days, and each experiment was performed in triplicate. The statistical significance was analyzed using ANOVA for each day, * indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$. For colony formation assay, six hundred Tca8113 cells and CAL27 cells were plated in 6-well plates. After a 14-day period, cells were washed with PBS, and fixed with 10% methanol for 15 min, and stained with Giemsa staining. Colony formation images were pictured for each well and the numbers of colony were counted with ImageJ software. All experiments were performed in triplicate. The statistical significance was analyzed using ANOVA, * indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$.

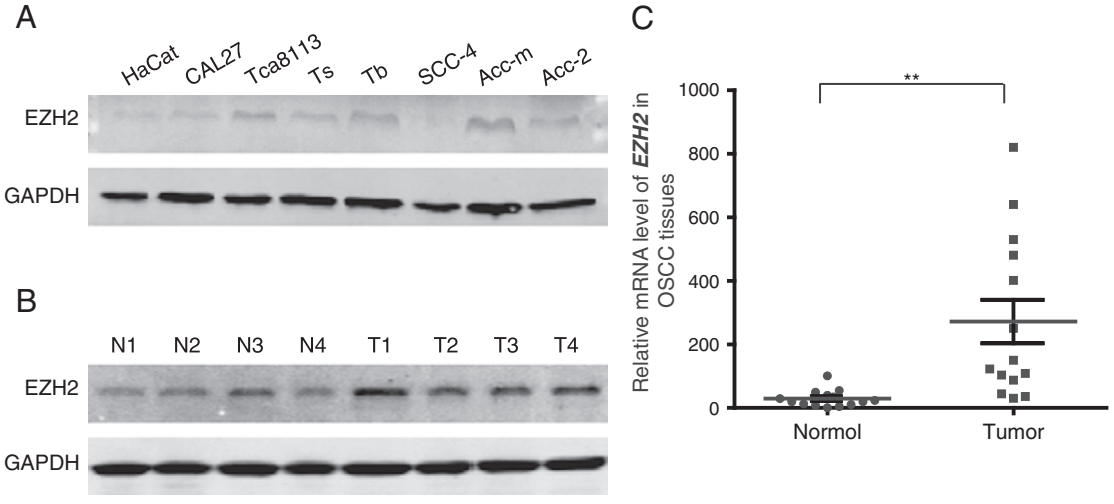

Fig. 1. EZH2 protein and messenger RNA (mRNA) levels are illustrated in OSCC cells and tissue specimens. (A) Cell extracts were prepared from HaCat, CAL27, Tca8113, Ts, Tb, SCC-4, ACC-M and ACC-2 cells, and analyzed by immunoblotting analyses with the anti-EZH2 antibody. GAPDH was detected as control. (B, C) EZH2 protein and mRNA levels were determined in 14 tissue specimens from patients with OSCC and in 4 paired OSCC tissue specimens using immunoblotting and qRT-PCR analyses. mRNA level was calculated by using the $2^{-\Delta\Delta Ct}$ method. ** indicates $P < 0.01$. GAPDH was detected as control.