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EZH2 promotes cell proliferation by regulating the expression of RUNX3 in laryngeal carcinoma

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Abstract Enhancer of zeste homolog 2 (EZH2) is a highly conserved histone methyltransferase, which is overexpressed in different types of cancers such as breast and prostate cancer. It is reported that EZH2 can directly down-regulate RUNX3 by increasing histone H3 methylation. However, the role of EZH2 in the development and progression of laryngeal carcinoma has not yet been investigated, and the relationship between EZH2 and RUNX3 in laryngeal carcinoma is rarely reported. The current study aims to determine the role of EZH2 in the progression of laryngeal carcinoma, and investigate the interaction between EZH2 and the tumor suppressor RUNX3. Our study found that EZH2 is overexpressed in laryngeal carcinoma patients, and silencing EZH2 by EZH2 siRNA significantly inhibited the proliferation of laryngeal carcinoma cells. Besides, we also found that RUNX3 is repressed in laryngeal carcinoma patients. Moreover, RUNX3 as a downstream target protein of EZH2 is up-regulated by EZH2 siRNA accompanied by a decrease in the trimethylation modification pattern of H3K27. RUNX3 siRNA inhibits the decreased proliferation induced by EZH2 siRNA. Furthermore, β-catenin protein expression is down-regulated by EZH2 siRNA and up-regulated by RUNX3 siRNA, and RUNX3 siRNA inhibits the down-regulation effect of EZH2 siRNA on β-catenin protein expression. Additionally, the Wnt/β-catenin activator BIO reverses the inhibitory effect of EZH2 siRNA on Hep-2 cell proliferation. Taken together, our results suggest that EZH2 regulates cell proliferation potentially by targeting RUNX3 through the Wnt/β-catenin signaling pathway in laryngeal carcinoma.

Keywords Enhancer of zeste homolog 2 (EZH2) · Laryngeal carcinoma · RUNX3 · Proliferation

Introduction

Laryngeal carcinoma is the second most common malignant type of head and neck tumors. It is estimated that laryngeal carcinoma accounted for approximately 12,630 new cases and about 3610 deaths in the United States in 2014 [1]. Despite advances having been achieved in diagnosis and treatment, the survival rate for laryngeal carcinoma patients has not advanced but has decreased over the last 30 years [2]. Therefore, a better understanding of the molecular basis of laryngeal carcinoma development and a new target for laryngeal carcinoma progression and treatment are urgently demanded.

Enhancer of zeste homolog 2 (EZH2) is the catalytic core protein of polycomb repressor complex 2 (PRC2) and a highly conserved histone methyltransferase which silences the expression of the target genes through catalyzing the trimethylation of lysine-27 of histone 3 (H3K27me3). Histone 3 has lysine residues at positions K4, K9, K27, and K36 that can undergo methylation [3]. Methylation can activate or suppress transcription depending on the histone residue that is methylated. Methylation of H3K9 and H3K27 is associated with silencing of transcription, while methylation at positions K4 and K36 is frequently related

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to activation of transcription [4, 5]. Three states of methylation including mono-, di-, and tri- are observed on H3K27. H3K27me3 is characteristic of PcG target genes and results in repression [6].

Overexpression of EZH2 is observed in different types of cancers, including breast, lung, prostate cancer, colorectal, and oral squamous cell carcinoma [7–9], and is always related to the progression of cancers. EZH2 alters the genomic expression program and is regarded as a valuable prognostic indicator of patient outcome in prostate cancer [10–12] and a prognostic indicator of outcome in breast cancer patients [13, 14]. It is reported that EZH2 is involved in the regulation of proliferation, cell cycle progression, and cell differentiation [7], and is suggested to be an oncogene that maintains DNA methylation and the stable repression of many tumor suppressor genes in cancer [8]. However, the role of EZH2 in the development and progression of laryngeal carcinoma has not yet been investigated.

Increasing evidence has indicated that EZH2 regulates tumor progression by regulating a series of target genes including E-cadherin [15], CDKN1C [16], and PSP94 [17], among others. Also, recent findings have implicated EZH2 in the direct down-regulation of RUNX3 by increasing histone H3 methylation [18], and have shown that EZH2 elevates cell proliferation by down-regulating the expression of RUNX3 in human cholangiocarcinoma [19]. RUNX3 belongs to the Runt family of transcription factors, which play important roles in both normal developmental processes and carcinogenesis [20]. RUNX3 is reported to be involved in the formation of a variety of cancers, and since being suggested as a tumor suppressor in gastric cancer for the first time [21], the anti-oncogene role of RUNX3 has been found in different types of cancers, including breast, liver, lung, bladder, and colorectal cancer, as a result of its frequent inactivation by promoter hypermethylation [18, 22]. RUNX3 is also known to be frequently silenced and hypermethylated, and has been suggested to be related to lymph node metastasis and the development of laryngeal carcinoma, while the relationship between EZH2 and RUNX3 in laryngeal carcinoma is rarely reported.

The present study aimed to determine the role of EZH2 in the progression of laryngeal carcinoma. We first determined the expression of EZH2 and RUNX3 in the tissues of laryngeal carcinoma patients, after which siRNA was used to study the role of EZH2 in the cell proliferation of laryngeal carcinoma in vitro. Furthermore, the interaction of EZH2 and tumor suppressor RUNX3 was investigated, and the involvement of Wnt/β-catenin in the regulation of EZH2 in laryngeal carcinoma is also estimated.

Materials and methods

Patients and specimens

Twenty-five larynx cancer tissues and 20 adjacent tissues were obtained from the First Affiliated Hospital of Xinxiang Medical University. None of the patients received any therapy before the surgery. Informed consent was obtained from all patients.

Cell culture

Hep-2 and TU212 cells were purchased from ATCC (Manassas, VA, USA) and cultured in RPMI 1640 medium (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (Gibco), 1% L-glutamine, and 1% penicillin–streptomycin (Sigma, St. Louis, MO, USA) in a humidified atmosphere incubator at 37 °C with 5% CO_2.

SiRNA interference

Hep-2 and TU212 cells were cultured and transfected with control siRNA and EZH2 siRNA (EZH2 siRNA1, 5-AAG AGGTTCAGACGAGCTGAT-3, and siRNA2, 5-AAG ACTCTGAATGCAGTTGCT-3) (Qiagen, Valencia, CA, USA) or RUNX3 siRNA (5′-TTTGCGGAGTAGTTC TCGTCATACAATGACGAHAACTACTCCGCTTTTT-3′, 5′-CTAGAAAAAGCGGAGTAGTTCTCGTCATTG TATGACGAGAACTACTCCG-3′ (Sangon Biotech, Shanghai, China) and its negative siRNA according to the manufacturer's protocol of lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Then, 48 h later, cells were collected and the mRNA expression of EZH2 and RUNX3 were measured using RT-PCR.

CCK-8 assay

Hep-2 and TU212 cells cultured in a 96-well plates with a density of 1×10^4 cells/well at 37 °C in a 5% CO_2 incubator for 24 h. Then, 10 μl CCK-8 solution was added to each well and incubated for 2 h. the optical density (OD) values were measured at 450 nm using an enzyme immunoassay analyzer (Bio-Rad Laboratories, Hercules, CA, USA).

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from Hep-2 cells or TU212 cells using Trizol reagent and synthesized cDNA using a Verso cDNA kit (Thermofisher Scientific). QRT-PCR was performed using a FastStart Universal SYBR Green Master

(Roche, Nutley, NJ, USA) on a Roche LightCycler480-II real-time thermal cycler (Roche). In brief, 45 μl reaction solutions containing 50 ng cDNA, 25 μl of FastStart Universal SYBR Green Master, 0.5 μl of forward primer and reverse primer, and 19 μl PCR-grade water were subjected to 95 °C for 10 min, and then 40 cycles of 95 °C for 15 s, 56 °C for 60 s, and 68 °C for 45 s. The primers used were as follows: EZH2 (Forward: TTGTTGGCG GAAGCGTG, Reverse: TCCCTAGTCCCGCGCAATG TGC); RUNX3 (Forward: GAGTTTCACCCTGACCAT-CACTGTG, Reverse: GCCCATCACTGGTCTTGAAGG TTG); and β-actin (Forward: TTCCTTCTTGGGTATG-GAAT, Reverse: GAGCAATGATCTTGATCTTC). Data were analyzed by $2^{-\Delta\Delta CT}$ method [23] and normalized by β-actin.

Western blot analysis

Total protein was extracted from Hep-2 cells or TU212 cells using RIPA (Beyotime, Nantong, China), and the concentrations were determined by Bradford reagent (Bio-Rad Laboratories, Hemel Hempstead, UK). Then, 10 μg total protein extract was used and separated by 10% SDS-PAGE gels and then transferred onto polyvinylidene difluoride membranes. After blocking membrane in 5% skim milk for 30 min, the rabbit polyclonal antibody RUNX3 and β-catenin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were added and incubated overnight at 4 °C. Then, HRP-conjugated goat anti-rabbit secondary antibodies (Santa Cruz Biotechnology) were added and incubated for 1 h at room temperature. The membranes were visualized with ECL systems (Amersham Biosciences, Chalfont St. Giles, UK). β-actin was used to normalize the data.

Chromatin immunoprecipitation (ChIP)

ChIP was carried out to determine the H3K27me3 in Hep-2 and TU212 cells according to the manuscript's protocols of Methyl-Histone H3K27 ChIP Kit (Epigentek, Farmingdale, NY, USA). Briefly, cells were collected and lysed; the cellular chromatin was fragmented by a sonication device and then added to the wells already covered with antibodies against H3K27me3. Chromatin fragments containing methylated lysine residues that bind to antibodies were attached to wells, while other fragments were removed from the bottom of wells by washing. The precipitated chromatin fragments were then detached from antibodies, and the DNA fragments were separated from histone proteins using the reverse cross-link procedure. The resulting DNA fragments were then purified and used to quantitatively analyze histone methylation pattern change using the RT-PCR assay. Specific primers designed for the RUNX3 gene promoter [18] are shown in Table 1, and the β-actin gene promoter was used as a housekeeping gene.

Statistical analysis

Data were processed using SPSS18.0 software and are expressed as mean ± SD. Differences between groups were assessed by *t* test; $P < 0.05$ was accepted as statistically significant.

Results

EZH2 is overexpressed and RUNX3 expression is down-regulated in laryngeal carcinoma tissues

To measure the expression level of EZH2 in laryngeal carcinoma, 25 laryngeal carcinoma tissues and 20 normal adjacent tissues were obtained from laryngeal carcinoma patients, and the mRNA expression of EZH2 was detected using RT-PCR analysis. The results showed that the mRNA expression level of EZH2 was detectable in 20 of the 25 laryngeal carcinoma tissues and 13 of the 20 adjacent tissues. The RT-PCR results showed that EZH2 expression level was significantly higher in laryngeal carcinoma than in the adjacent tissues among the detectable tissues ($P < 0.05$, Fig. 1a). Meanwhile, the mRNA expression level of RUNX3 was remarkably lower in tumor tissues than adjacent tissues ($P < 0.05$, Fig. 1b), which shows a contrasting tendency to EZH2. Besides, the correlation analysis between the EZH2 expression and RUNX3 expression in tumor tissues showed that there is a negative relationship between EZH2 and RUNX3 ($P < 0.05$, Fig. 1c).

Knockdown of EZH2 inhibits the cell proliferation in larynx carcinoma

To determine the role of overabundant EZH2 in the progression of laryngeal carcinoma, laryngeal carcinoma cell lines Hep-2 and TU212 were cultured and transfected with EZH2 siRNA, and cell proliferation was assessed by CCK-8 assay. The results showed that EZH2 siRNA significantly repressed the mRNA and protein expression of EZH2 in Hep-2 and TU212 cells ($P < 0.05$, Fig. 2a, b). The proliferation of both Hep-2 and TU212 cells was statistically significant inhibited by EZH2 siRNA in a time-dependent manner ($P < 0.05$, Fig. 2c, d). With increasing time, the effect of EZH2 siRNA on proliferation was significant after 48 h ($P < 0.05$, Fig. 2c, d).

Table 1 Primer sequences used for CHIP

Genes	Forward primer	Reverse primer
RUNX3	TGTCCCGGGATCCTCTTCT	ATGAGACGTTGGTGCCC
β-actin	CCCTGGCGGCCTAAGGACTC	CACATGAAGGAGCCGTTGTC

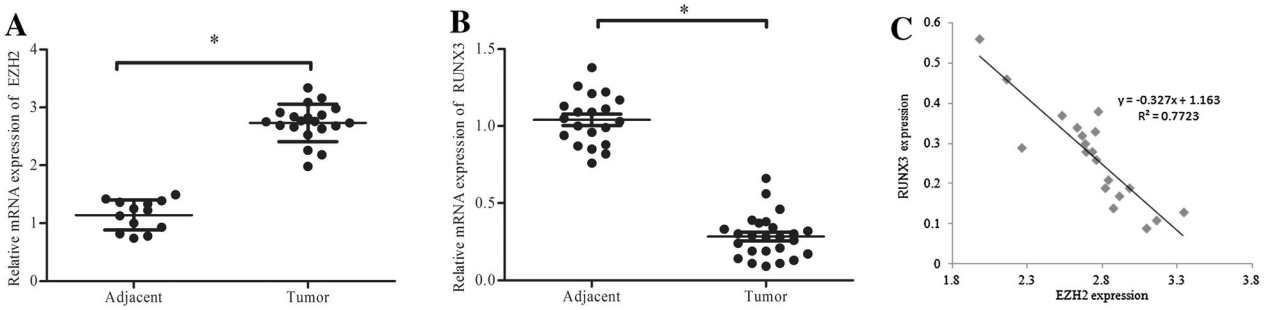

Fig. 1 The mRNA expression level of EZH2 and RUNX3 in laryngeal carcinoma tissues and adjacent tissues. **a** RT-PCR analysis of mRNA expression levels of EZH2 in 25 laryngeal carcinoma tissues and 20 adjacent tissues. **b** RT-PCR analysis of mRNA expression levels of RUNX3 in 25 laryngeal carcinoma tissues and 20 adjacent tissues. **c** The correlation analysis between EZH2 expression and RUNX3 expression in laryngeal carcinoma tissues. $*P < 0.05$ versus adjacent

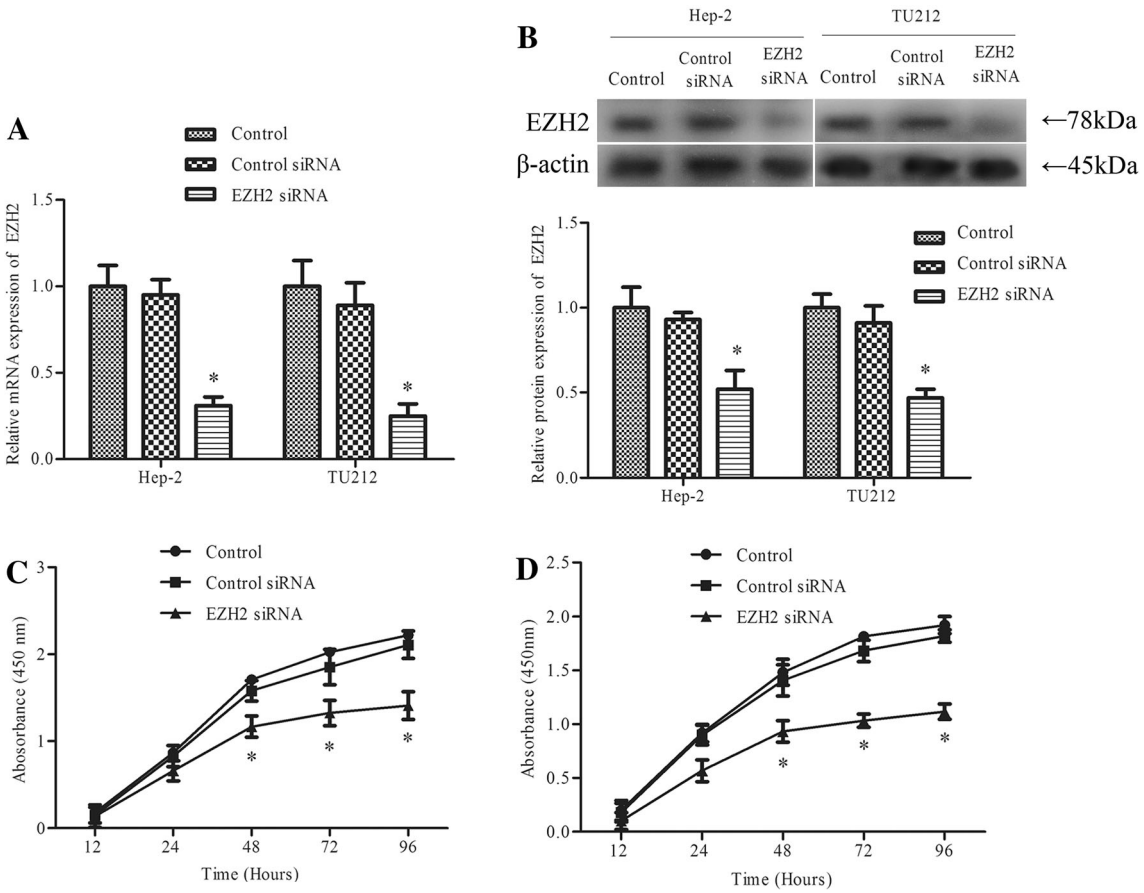

Fig. 2 Knockdown of EZH2 promotes cell proliferation in laryngeal carcinoma cell lines. **a** The mRNA expression level of EZH2 in Hep-2 and TU212 cells transfected with EZH2 siRNA was measured by RT-PCR. **b** The protein expression level of EZH2 in Hep-2 and TU212 cells transfected with EZH2 siRNA was measured by western blot. **c** The proliferation of Hep-2 cells transfected with EZH2 siRNA was determined by CCK-8 assay. **d** The proliferation of TU212 cells transfected with EZH2 siRNA was determined by CCK-8 assay. The results are from six replicate samples (±SD). $*P < 0.05$ versus control

EZH2 regulates the expression of RUNX3

The above results show that the expression levels of EZH2 and RUNX3 showed a contrary tendency in tissues from laryngeal carcinoma patients, so the relationship between EZH2 and RUNX3 was studied in the present study. EZH2 siRNA was used to knock down EZH2, and the expression level of RUNX3 was assessed in Hep-2 and TU212 cell lines. The results showed that EZH2 siRNA up-regulates the mRNA expression level of RUNX3 remarkably both in Hep-2 and TU212 cells ($P < 0.05$, Fig. 3a). The RUNX3 protein expression levels were also significantly up-regulated by EZH2 siRNA ($P < 0.05$, Fig. 3b).

As EZH2 is a highly conserved histone methyltransferase which always silences gene expression by catalyzing H3K27me3, we assessed the epigenetic mechanisms of the down-regulation effect of EZH2 on RUNX3; the H3K27me3 of RUNX3 was detected using EpiQuik™ Methyl-Histone H3K27 ChIP Kit. As shown in Fig. 3, H3K27me3 was found in the promoter of RUNX3 in Hep-2 cells, and showed a 0.54-fold decrease in EZH2 siRNA transfected Hep-2 cells ($P < 0.05$, Fig. 3c).

EZH2-induced cell proliferation is potentially associated with repression of RUNX3 expression

As RUNX3 is one of the targets of EZH2, we evaluated the role of RUNX3 in the regulation of EZH2 on the proliferation in Hep-2 cells. RUNX3 was silenced by RUNX3 siRNA ($P < 0.05$, Fig. 4a, b), RUNX3 siRNA can significantly promote the proliferation, and the inhibitory effect of EZH2 siRNA on the proliferation was significantly attenuated by RUNX3 siRNA ($P < 0.05$, Fig. 4c).

Wnt/β-catenin signaling pathway is involved in the regulatory effect of EZH2 on cell proliferation

It is well-known that the Wnt/β-catenin pathway has essential functions in the regulation of cell growth and differentiation. To further explore the molecular mechanism of EZH2 knockdown inhibited cell proliferation, the role of the Wnt/β-catenin signaling pathway was studied. The results showed that EZH2 siRNA could significantly down-regulate the protein expression of β-catenin, while RUNX3 siRNA and the Wnt/β-catenin activator BIO

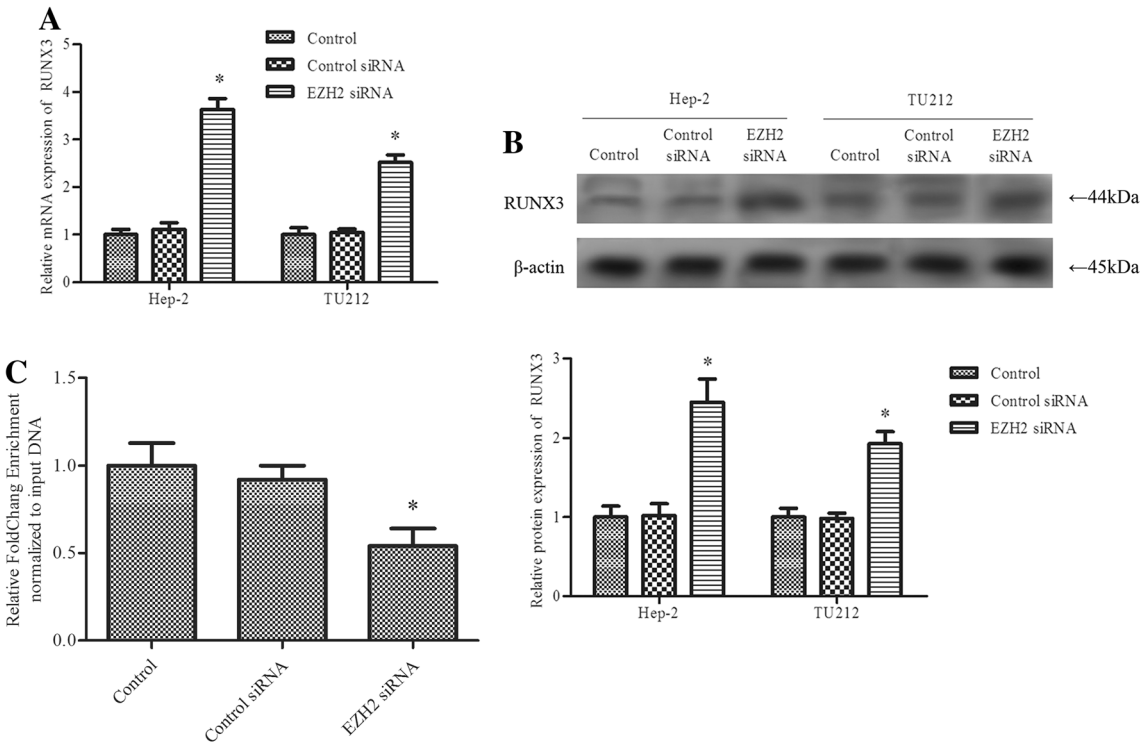

Fig. 3 EZH2 regulates the expression of EZH2. **a** The mRNA expression level of RUNX3 in Hep-2 and TU212 transfected with EZH2 siRNA was measured by RT-PCR. **b** The protein expression levels of EZH2 in Hep-2 and TU212 cells transfected with EZH2 siRNA were measured by western blot. **c** H3K27me3 on the promoter of RUNX3 gene was analyzed by Methyl-Histone H3K27 ChIP Kit. The results are from six replicate samples (±SD). *$P < 0.05$ versus control

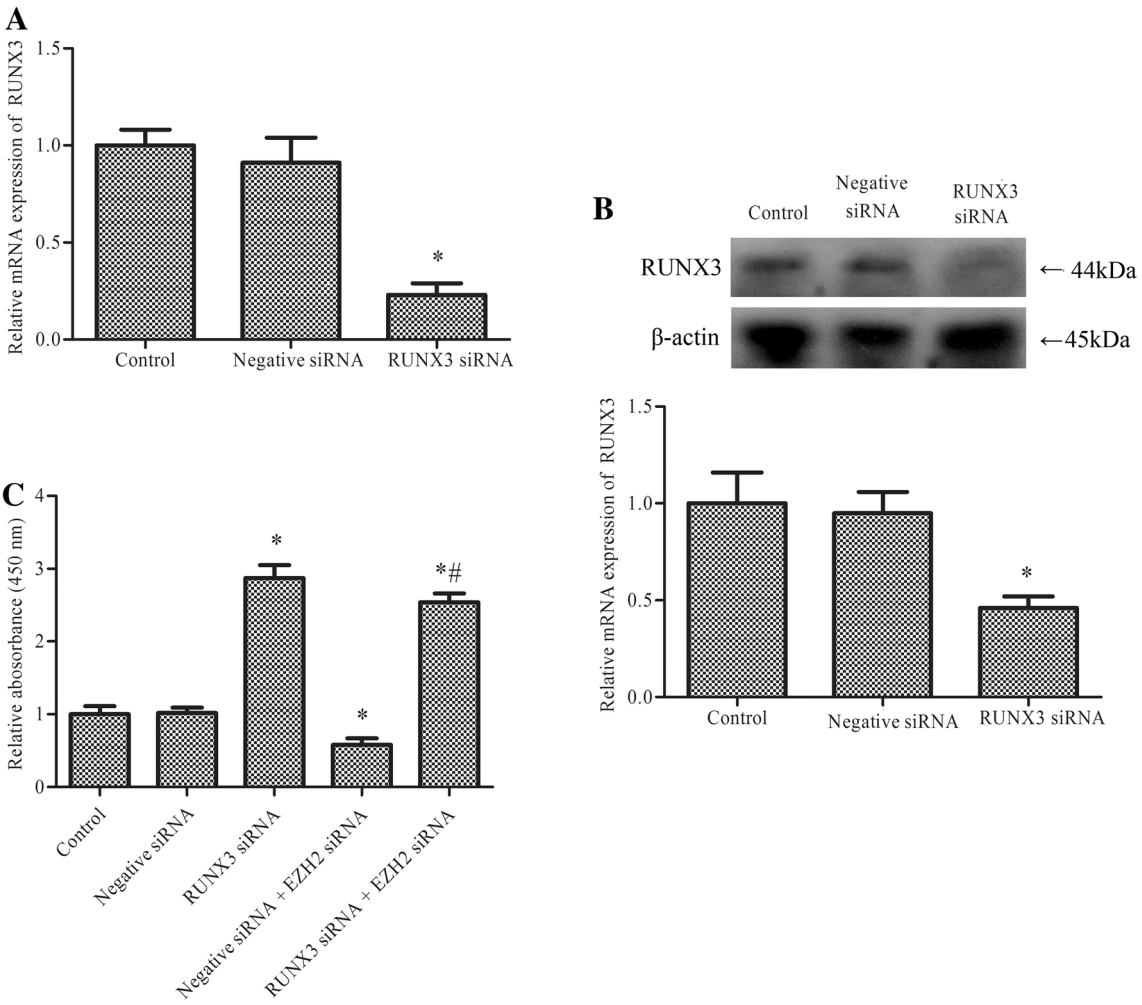

Fig. 4 RUNX3 is involved in the regulatory effect of EZH2 on cell proliferation. **a** The mRNA expression level of RUNX3 in Hep-2 cells transfected with RUNX3 siRNA was measured by RT-PCR. **b** The protein expression level of RUNX3 in Hep-2 cells transfected with RUNX3 siRNA was measured by western blot. **c** Cell proliferation was detected by the CCK-8 assay in Hep-2 cells transfected with RUNX3 siRNA or co-transfected with RUNX3 siRNA and EZH2 siRNA. The results are from six replicate samples (±SD). $^{*}P < 0.05$ versus control. $^{\#}P < 0.05$ versus EZH2 siRNA

significantly up-regulated β-catenin protein expression in Hep-2 cells; furthermore, RUNX2 siRNA could significantly inhibit the down-regulation effect of EZH2 siRNA on the β-catenin protein expression ($P < 0.05$, Fig. 5a). The results of the CCK-8 assay showed that BIO significantly promoted the proliferation and EZH2 siRNA inhibited the cell proliferation of Hep-2 cells, while RUNX3 siRNA or BIO could significantly reverse the inhibitory effect of EZH2 siRNA on Hep-2 cell proliferation ($P < 0.05$, Fig. 5b).

Discussions

EZH2 is a mammalian histone methyltransferase that contributes to the epigenetic silencing of target genes and regulates the survival and metastasis of cancer cells. Increased EZH2 expression has been found in diverse malignancies, and is associated with tumor cell cycle and cell proliferation [7, 24]. The present study investigated the expression of EZH2 in laryngeal carcinoma for the first time, and found that EZH2 was overexpressed in laryngeal carcinoma patients. Furthermore, we also found that knockdown of EZH2 inhibited cell proliferation in Hep-2 and TU212 cells, which is consistent with a growth-promoting role of EZH2 in prostate cancer, breast cancer, and colon cancer [13, 25, 26].

The molecular mechanisms for EZH2-stimulated cell proliferation have been studied in some tumors, and the repression of anti-proliferation genes through epigenetic silencing is regarded as one of the most important molecular events. Several proliferation-related genes such as the p27 cell cycle regulator gene BRCA1 are proven targets of EZH2 [27, 28]. Fujii et al. [18]. suggested that RUNX3 is

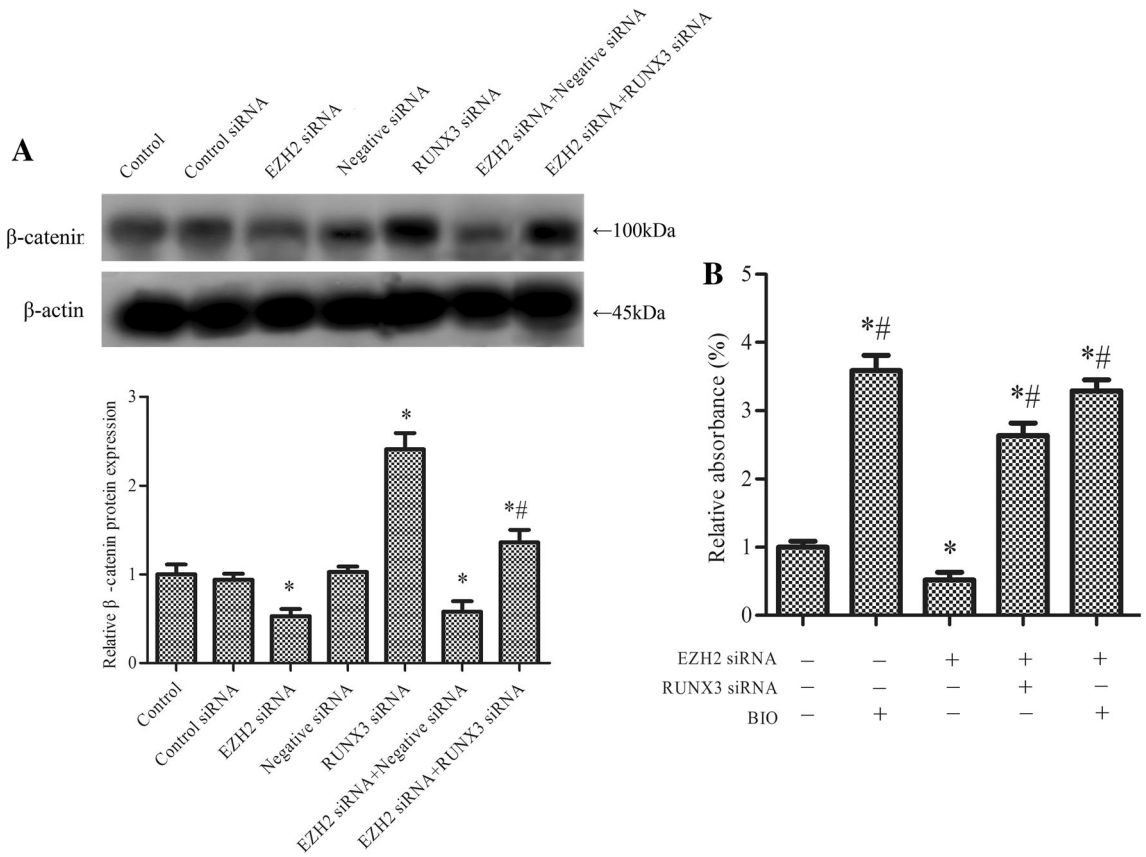

Fig. 5 Wnt/β-catenin signaling pathway is involved in the regulation effect of EZH2 on cell proliferation. **a** The β-catenin protein expression levels were measured by western blot in Hep-2 cells transfected with EZH2 or RUNX3 or treated with the Wnt/β-catenin activator BIO. **b** Cell proliferation was determined by the CCK-8 assay in Hep-2 cells transfect with EZH2 siRNA or RUNX3 siRNA or treated with BIO. The results are from six replicate samples (±SD). $^{*}P < 0.05$ versus control. $^{\#}P < 0.05$ versus EZH2 siRNA transfection only

also a target for repression by EZH2 through increasing histone H3 methylation in gastric, breast, prostate, colon, and pancreatic cancer cell lines, and the repressed RUNX3 might be a mechanism underlying the proliferative effects of EZH2. The RUNX3 gene is located on 1p36, a region that is often deleted, and thus believed to harbor tumor suppressor genes in a wide variety of human carcinomas, including pancreatic and lung cancer [29, 30]. Inactivation of RUNX3 has been frequently found in various types of human cancers, and acts as a tumor suppressor [31–33]. Our study found that the expression of RUNX3 was significantly suppressed in laryngeal carcinoma tissues. These results were consistent with the results of Xu et al. [34], who found that RUNX3 expression was down-regulated in both hypermethylated and unmethylated laryngeal carcinoma tissues. However, Kim et al. [35]. also reported that RUNX3 methylation was detected in 62% of the 37 larynx cancers studied and increasing evidence has indicated that the frequent inactivation of RUNX3 is due to DNA hypermethylation in gastric cancer, prostate cancer, breast cancer, colon cancer, hepatocellular carcinoma. Taking together, we suggested that the aberrant methylation of CPG islands was just one important potential mechanism for the inactivation of RUNX3, and that there might be other mechanisms responsible for the down-regulation of RUNX3 in LSCC.

As a polycomb-group protein involved in the epigenetic silencing of genes, EZH2 regulates a large set of tumor suppressor genes in cancers [36]. It has been reported that EZH2 down-regulates RUNX3 by increasing histone H3 methylation, and that may be one pathway by which EZH2 affects tumor cell proliferation [18]. In the present study, we found that EZH2 siRNA, which knocks down EZH2, promoted the expression of the RUNX3 gene in laryngeal carcinoma cells without any change in the DNA methylation of the RUNX3 promoter region, but significantly decreased H3K2me3. This finding suggested that H3K27me3 of RUNX3 by EZH2 plays a key role in the down-regulation of RUNX3, rather than DNA methylation in the promoter. Further investigation found that RUNX3 was a target of EZH2, promoting proliferation in Hep-2 and TU212 cells.

The Wnt/β-catenin signaling pathway is deregulated and plays an important role in regulating cell proliferation in many types of cancer [37–39]. Cheng et al. [40]. revealed that the overexpression of EZH2-activated Wnt/β-catenin signaling promotes cellular proliferation in hepatocellular carcinomas. Our study showed that knockdown of EZH2 impedes the protein expression of β-catenin, while RUNX siRNA inhibits the down-regulation effect of EZH2 siRNA on the β-catenin protein expression, and BIO attenuates the proliferative effect in laryngeal carcinoma; these results imply that RUNX3 is a downstream target protein that regulates the Wnt/β-catenin signaling pathway and that Wnt/β-catenin signaling is an important pathway by which EZH2 regulates cell proliferation in laryngeal carcinoma.

In summary, our study suggests that EZH2 is overexpressed and has a crucial role in cell proliferation potentially by regulating RUNX3 through the Wnt/β-catenin signaling pathway in laryngeal carcinoma. A specific therapeutic strategy based on the inhibition of EZH2 may be useful in the treatment of laryngeal carcinoma.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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