EZH2 promotes invasion and tumour glycolysis by regulating STAT3 and FoxO1 signalling in human OSCC cells

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Abstract
The enhancer of zeste homolog 2 (EZH2), known as a member of the polycomb group (PcG) proteins, is an oncogene overexpressed in a variety of human cancers. Here, we found that EZH2 correlated with poor survival of oral squamous cell carcinoma (OSCC) patients using immunohistochemistry staining. EZH2 overexpression led to a significant induction in tumour glycolysis, Epithelial-mesenchymal transition (EMT), migration and invasion of OSCC cells. Conversely, silencing of EZH2 inhibited tumour glycolysis, EMT, migration and invasion in OSCC cells. Ectopic overexpression of EZH2 increased phosphorylation of STAT3 at pY705 and decreased FoxO1 expression, and FoxO1 expression was enhanced when inhibiting STAT3. In addition, EZH2 overexpression led to a significant decrease in FoxO1 mRNA levels in nude mice xenograft. These results indicated that regulation of EZH2 might have the potential to be targeted for OSCC treatment.

Keywords
enhancer of zeste homolog 2, epithelial-mesenchymal transition, FoxO1, invasion, oral squamous cell carcinoma, STAT3, tumour glycolysis
1 | INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most common epithelial malignancy in oral cavity, with 354,864 new cases diagnosed worldwide and 177,384 deaths every year. Despite advances in cancer management, the overall 5-year survival rate of OSCC patients has remained less than 50% and has not improved significantly during the past three decades. Major reason for this circumstance is the tendency of OSCC to invade adjacent tissues and metastasis, with the local and regional recurrence rate ranging from about 33% to 40%. More detailed studies are required to understand the mechanisms mediating invasion and metastasis of OSCC in order to prolong patients’ survival.

Epithelial-mesenchymal transition (EMT), during which the epithelial cells obtain a mesenchymal cell phenotype with enhanced migratory and invasive capacities, plays pivotal roles in organ development, wound healing and cancer metastasis. Recent studies implied that EMT may have a close relationship with tumour glycolysis. Tumour glycolysis, which is also known as the Warburg effect and widely recognized as a central hallmark of cancer, refers to a phenomenon whereby most cancer cells metabolize glucose via fermentation even in the presence of oxygen and could be increased to meet the enhanced bioenergetics and biosynthetic requirements of cancer cells during EMT. In colorectal carcinoma, tumour glycolysis and EMT could be induced by deficiency of α/β-hydrolase domain-containing-5 (Abhd5), an intracellular lipolytic activator, thus promoting EMT and cancer metastasis. Interestingly, a recent research has reported that EZH2 expression was decreased under the condition of glycolysis restriction in effector T cells mediated by ovarian cancers, implying the possible relationship between EZH2 and glycolysis. Therefore, we hypothesize that EZH2 might take part in the invasion and tumour glycolysis of OSCC cells.

In the present study, we demonstrated that aggressive behaviour was positively associated with the mRNA and protein expressions of EZH2 in OSCC clinical samples, OSCC cell lines and subcutaneous xenograft models of nude mice. Then, we observed that overexpression of EZH2 promoted EMT and invasion mediated by tumour glycolysis in OSCC cells through regulating STAT3 and FoxO1 signalling pathway. Taken together, these data reveal the mechanism by which EZH2 regulates OSCC EMT and invasion through tumour glycolysis and, further, suggest that the regulation of EZH2 expression may be a promising target for OSCC therapeutics.

2 | MATERIALS AND METHODS

2.1 | Reagents

Monoclonal mouse anti-human antibodies to EZH2 (sc-166609), STAT3 (sc-293151), FoxO1 (H-128) and β-catenin (sc-1496) were obtained from Santa Cruz Biotechnology Inc. Monoclonal mouse anti-human antibodies to tyrosine-phosphorylated STAT3 (pY-STAT3, at the 705 residue) (9145S) and E-cadherin (3195S) were purchased from Cell Signaling Technology Inc. Monoclonal mouse anti-human antibodies to N-cadherin (ab98952) and vimentin (ab8978) were obtained from Abcam. Monoclonal mouse anti-human antibody to β-actin (ab008-100) was obtained from MultiSciences. 2-deoxy-glucose (2-DG) was purchased from Sigma, and cells were treated with 5 mmol/L 2-DG for 48 hours before further research.

2.2 | Patient specimens

Sixty-eight cases of OSCC tissues and 10 cases of normal oral mucosa between 2000 and 2003 were obtained from Department of Oral and Maxillofacial Surgery, West China School of Stomatology, Sichuan University. Tissues were fixed in 10% formalin and embedded in wax. The histological features and cell differentiation-based OSCC grading of all tumours were classified according to the current Union for International Cancer Control (UICC) criteria. The informed consent was obtained from all cases, and the study was carried out...
according to the World Medical Association Declaration of Helsinki. This study was approved by the Institutional Ethics Committee of the West China Medical Center, Sichuan University, China.

### 2.3 Immunohistochemistry

Paraffin-embedded blocks were sectioned in 4 μm slices. After deparaffinization and rehydration, antigen retrieval was performed using 0.01 mol/L citrate buffer (pH = 6) in an autoclave for 5 minutes. The sections were incubated with 3% hydrogen peroxide and normal goat serum working fluid for 15 minutes consecutively and then immunostained with monoclonal mouse anti-human antibodies to EZH2 at a 1:150 dilution at 4°C overnight. After washed in PBS, the sections were incubated with secondary antibody for 15 minutes. The sections were incubated with 3% hydrogen peroxide and using 0.01 mol/L citrate buffer (pH = 6) in an autoclave for 5 minutes. Antigen retrieval was performed by three of us independently who were blind to clinical outcome and sections were counterstained with haematoxylin.

Quantification of immunohistochemical results was performed by three of us independently who were blind to clinical outcome and six microscopic fields at 200× magnification per section were observed. The ratio of positive cells was counted in 1000 random cells in every field, and the mean percentage per section was categorized. EZH2 expression was classified into lower (positive cells were <50%) and higher (positive cells were 50% or more) based on the median of percentage for all cases. For representing negative expression, weakly positive expression, moderately positive expression and strongly positive expression, the percentage was categorized into (-), <5%; (+), 5%-25%; (++), 25%-50%; (+++), >50%.

### 2.4 Cell Culture

Human squamous cell carcinoma of tongue cancer cell lines Cal-27 and Tca8113 was provided by State Key Laboratory of Oral Diseases, Sichuan University. All cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS in a humidified atmosphere of 95% air and 5% CO₂ at 37°C.

### 2.5 Plasmids and shRNAs

The human EZH2 gene stably overexpressing vector using vector pDC316-mCMV-mCherry-Puro was constructed by Hibio (Hangzhou, China). The shRNA lentiviral vector pHBLV-U6-mCherry-Puro was from Hibio. Targeted sequences of shRNAs were as follows: EZH2: GCAAAATTTCTCGGTGTCAAAAA (sh-1-E), GGATGGTACTTTCATTGAA (sh-2-E) and TCGGTAAATCCAAACTGCTATGCAA (sh-3-E); STAT3: GCAAATTCTCGGTGTCAAA (sh-1-E), GGATGGTACTTTCATTGAA (sh-2-E) and TCGGTAAATCCAAACTGCTATGCAA (sh-3-E); scramble: TTCTCCGAACGTGTCACGTAA. All constructs were verified by sequencing.

### 2.6 Transfections

Oral squamous cell carcinoma cell lines were seeded at 20% confluence in 12-well plates the day before transfection. The lentiviral particles of EZH2 or shRNAs were added to target cells in the presence of 5 μg/mL of polybrene and incubated for 12 hours. Transfected cells were selected with 2 μg/mL puromycin for 7 days. The expressions of the target genes were verified by Western blot and RT-PCR analysis.

### 2.7 RT-PCR

Total RNA was prepared using TRIzol reagent (Invitrogen) following the manufacturer’s instructions. The reverse transcription was carried out using HiScript II Q RT SuperMix for qPCR (Vazyme). Quantitative real-time PCR was performed with ChamQ™ SYBR Color qPCR Master Mix (Vazyme) on CFX Connect Real-Time System (Bio-Rad) according to manufacturer’s instructions. Specific primers used for RT-PCR were listed in Supplementary Data. Results were represented using 2-ΔΔCt method.

### 2.8 Western blot

Cells were lysed using 200 μL RIPA lysis buffer (Santa Cruz) for 30 minutes. Samples were then separated on SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked in normal goat serum for 2 hours at room temperature. Then, the membranes were probed with primary antibody to EZH2, STAT3, pY-STAT3, FoxO1, E-cadherin, N-cadherin, β-catenin, vimentin or β-actin at a 1:1000 dilution overnight at 4°C, followed by the incubation with goat antimouse antibody (MultiSciences) used at a 1:5000 dilution for 1 hour at room temperature. The interaction was detected by chemiluminescence (ECL) reagent (Beyotime Biotechnology, Shanghai, China) and visualized with ChemiDoc XRS + System (Bio-Rad). Antibody to β-actin was used to detect the loading amount.

### 2.9 Wound healing assay

Cells were seeded in 6-well plates at 5.0 × 10⁵ cells/well. When cells formed confluent monolayers, individual wells were scratched with a pipette tip to form a gap space. PBS was used to wash out the cell debris. Cells were incubated with medium containing no FBS. Photomicrographs were taken at 0, 24 and 36 hours. The closed scratch areas were measured using ImageJ software. Experiments were carried out in triplicate.

### 2.10 Cell invasion assay

Cells were starved in serum-free DMEM for 16 hours and then seeded in the upper chambers of 24-well plates (pore size 8 μm; Millipore) at 5.0 × 10⁴ cells/well coated with Matrigel (BD Bioscience). DMEM with 10% FBS was added to the lower chambers. After 24 hours incubation, the invasive cells stained with 0.1% crystal violet were counted using a microscope in five pre-determined fields (×200). Each assay was carried out in triplicate.
2.11 | Immunofluorescence staining

Cells were treated with E-cadherin, N-cadherin, β-catenin and vimentin primary antibodies overnight at 4°C, followed by the incubation with Alexa Fluor 488 chicken antimouse IgG (H + L) (A21200; Invitrogen) for 1 hours at room temperature. Nuclei were stained using DAPI solution (Sigma-Aldrich). Finally, images were captured using a fluorescence microscope (Olympus BX51).

2.12 | Flow cytometry-based apoptosis analysis

Cells were grown in 6-well plates and digested after 48 hours. For cell apoptosis measurement, the cells were resuspended in 1 × Binding Buffer, and 5 µL of Annexin FITC Conjugate and 10 µL of Propidium Iodide Solution were added into each cell suspension, separately. The stained cells were then analysed with a flow cytometry (FACScalibur, Becton-Dickinson).

2.13 | Glucose Consumption and Lactate Production Assays

Glucose (Rongsheng Biotechnology) and lactate (Abcam) assay kits were used to detect the glucose consumption and lactate production levels according to the manufacturer’s instructions. Results were normalized to $10^5$ cells.

2.14 | Subcutaneous xenograft model of nude mice

All animal experimental studies were approved by Sichuan University Animal Care and Use Committee. Twelve 4-week-old BALB/c male nude mice were purchased from the Slaccas experimental animal company. After 1 week acclimation, nude mice were divided into two groups randomly. Stably EZH2 overexpressed Cal-27 cells and control cells transfected with empty vectors were inoculated into nude mice separately by subcutaneous injection into the right flank region. Each mouse was performed with aliquots of 0.1 mL containing $5.0 \times 10^6$ cells per aliquot. Fluorescence in vivo images were taken to observe the tumour at day 29 using an IVIS Lumina XRMS Series III (Caliper Life Sciences). Tumour volumes were measured 3× per week and calculated using the formula: length × (width)$^2$ × π/6. Mice were killed at day 31. Tumours were collected for further examination, and tumour weights and volumes were measured. The present study was approved by the Institutional Animal Care and Use Committee of the West China Medical Center, Sichuan University, China.

FIGURE 1 Enhancer of zeste homolog 2 (EZH2) overexpression correlates with the metastasis and poor prognosis of oral squamous cell carcinoma (OSCC) patients. A, Tissue sections of representative OSCC sample and normal oral mucous stained for EZH2 by immunohistochemistry were shown. Magnification: Left 100×, Right 400×. B, The distribution of OSCC patients with different positive rates of EZH2 expression. For representing negative expression, weakly positive expression, moderately positive expression and strongly positive expression in immunohistochemistry results of OSCC specimens, the percentage was categorized into (−), <5%; (+), 5%–25%; (++), 25%–50%; (+++), >50%. C, Kaplan-Meier survival analysis of patients with OSCC showed that higher expressions of EZH2 were associated with a shorter overall survival rate.
2.15 Statistical analysis

All values were expressed as means ± SD. Data were analysed using GraphPad Prism 7.0 (GraphPad Software). The Student t test, one-way ANOVA and chi-square test were used to analyse the statistical differences. The Kaplan-Meier method was applied for the overall survival, and long rank test was used to evaluate statistical significances between groups. The multivariate analysis was performed by a Cox proportional hazards model to examine the potential prognostic factors, and P < .05 was considered statistically significant.

3 RESULTS

3.1 EZH2 overexpression correlates with the metastasis and poor prognosis of OSCC patients

Immunohistochemical staining of EZH2 was performed in 68 human OSCC samples and 10 normal human oral mucosae. As shown in Figure 1A, immunoreactivity for EZH2 was observed in the nuclei of OSCC cells and normal epithelial cells. EZH2 was expressed in 92.6% (63/68) cases of OSCC specimens and 20% (2/10) cases of normal oral mucosae (P < .0001). The weakly,
TABLE 3  Multivariate survival analysis for prognostic factors of oral squamous cell carcinoma (OSCC) patients using a Cox regression

|                | B   | SE  | Wald  | df | Sig. | Exp (B) | 95.0% CI for Exp(B) |
|----------------|-----|-----|-------|----|------|---------|---------------------|
|                |     |     |       |    |      |         | Lower              |
| EZH2           | -1.550 | 0.771 | 4.038 | 1  | .044 | 0.212   | 0.047              |
| Tumour size    | -1.082 | 0.465 | 5.418 | 1  | .020 | 0.339   | 0.136              |
| Differentiation|     |     |       |    |      |         | 0.843              |
| Well           | Reference |     |       |    |      |         |                     |
| Moderate       | -1.100 | 0.582 | 3.578 | 1  | .059 | 0.333   | 0.106              |
| Poor           | -13.414 | 303.300 | 0.002 | 1  | .965 | 0.000   | 2.207E + 252       |
| Clinical stage | -1.956 | 0.795 | 6.053 | 1  | .014 | 0.141   | 0.030              |
| Nodal metastasis| -0.380 | 0.440 | 0.744 | 1  | .389 | 0.684   | 0.289              |

FIGURE 2  Enhancer of zeste homolog 2 (EZH2) overexpression enhances migration, invasion potential and EMT in oral squamous cell carcinoma (OSCC) cells. A, Cal-27 and Tca8113 cells with stable EZH2 overexpression or control cells were established and subjected to RT-PCR and Western blot analysis. B, Wound healing assays of EZH2-overexpression cells and control cells. Shown were representative images on the left and quantitative assays on the right. The closed scratch area (%) calculated by ImageJ software was quantified in wound healing assay. C, Transwell invasion assays of EZH2-overexpression cells and control cells. Quantification of invasive cells through Matrigel of each cell line was shown as proportions of their controls. Invasive cells in five pre-determined fields were counted. D, Relative expressions of E-cadherin and β-catenin, and N-cadherin and vimentin were examined in Cal-27/EZH2 cells and control cells using Western blot and RT-PCR. E, Immunofluorescence staining was used to examine expressions of E-cadherin, β-catenin, N-cadherin and vimentin (green) in Cal-27/EZH2 and control cells. Nuclei were stained with DAPI (blue). F, Flow cytometry was used to examine the percentage of apoptotic cells in Cal-27/EZH2 and control cells. All assays were carried out in triplicate. Results were shown as means ± SD. *P < .05; **P < .01; ***P < .001

moderately and strongly positive rates of EZH2 in OSCC specimens were 11.8% (8/68), 29.4% (20/68) and 51.5% (35/68), respectively (Table 1, Figure 1B). Then, these immunohistochemistry results and their associations with corresponding clinicopathological features in 68 OSCC patients were summarized. We chose 50% (nearly the median for all cases in our study) as the cut-off point to
differentiate EZH2 expression into lower and higher part. Results showed that higher EZH2 expression was significantly associated with higher tumour size \( (P = .019) \), poorer differentiation \( (P = .006) \), higher clinical stage \( (P = .023) \) and the presence of nodal metastasis \( (P = .012) \), but not with age, gender, drink, smoke and recurrence \( (P = .012) \). These data were also obtained in the negative, weakly, moderately and strongly positive rate of EZH2 in OSCC specimens \( (P = .012) \). To further evaluate the effects of EZH2 on prognosis, survival curves were plotted using the Kaplan-Meier method and log-rank test \( (P = .012) \). The patients with higher EZH2 expression had a poorer prognosis than those with lower expression \( (P = .016) \). Multivariate survival analysis for prognostic factors using a Cox regression revealed that EZH2 expression, tumour size and clinical stage were independent prognostic factors \( (P = .044, P = .020; \text{Table 3}) \). These indicated that EZH2 overexpression significantly associated with metastasis and poor prognosis of OSCC patients.

### 3.2 EZH2 overexpression enhances migration, invasion potential and EMT in OSCC cells

To further investigate the role of EZH2 in the migration, invasion and EMT of OSCC cells, we assessed the relative EZH2 mRNA expression in five OSCC cell lines \( (P = .044) \). We established stable EZH2 overexpression transfectants in Cal-27 and Tca8113. RT-PCR and Western blot analysis demonstrated that EZH2 stably overexpressed cells enhanced the protein and mRNA expressions of EZH2 \( (P = .016) \). Then, we performed wound healing and transwell invasion assays, and the results showed that the abilities of migratory and invasive cells in Cal-27/EZH2 and Tca8113/EZH2 groups were significantly enhanced compared to the scrambled control groups.}

**FIGURE 3** Enhancer of zeste homolog 2 (EZH2) knockdown inhibits migration, invasion potential and EMT in oral squamous cell carcinoma (OSCC) cells. A, Cal-27 and Tca8113 cells with stable EZH2 knockdown or scrambled control cells were established and subjected to RT-PCR and Western blot analysis. B, Wound healing assays of EZH2-knockdown cells and scrambled control cells. Shown were representative images on the left and quantitative assays on the right. C, Transwell invasion assays of EZH2-knockdown cells and scrambled control cells. Quantification of invasive cells through Matrigel of each cell line was shown as proportions of their controls. D, Relative expressions of E-cadherin and \( \beta \)-catenin, and N-cadherin and vimentin were examined in Cal-27 cells with EZH2 knockdown and scrambled control cells using Western blot and RT-PCR. E, Immunofluorescence staining was used to analyse expressions of E-cadherin, \( \beta \)-catenin, N-cadherin and vimentin \( (\text{green}) \) in Cal-27 cells with EZH2 knockdown and scrambled control cells. Nuclei were stained with DAPI \( (\text{blue}) \). F, Flow cytometry was used to examine the percentage of apoptotic cells in Cal-27 cells with EZH2 knockdown and scrambled control cells. All assays were carried out in triplicate. Results were shown as means \( \pm \) SD. \( \ast P < .05; \ast \ast P < .01; \ast \ast \ast P < .001 \)
increased compared with those in the control group (Figure 2B and 2C). EZH2 overexpression increased the migrated area by 32.4% and led to a 0.7-fold increase in the cell invasion in Cal-27/EZH2 group, and increased the migrated area by 36% and led to a 0.9-fold increase in the cell invasion in Tca8113/EZH2 group (Figure 2C).

As EMT plays a critical role in the migration and invasion of epithelial cancer cells, we then examined both the epithelial and mesenchymal markers by Western blot, immunofluorescence and RT-PCR in Cal-27 cells. As can be seen in Western blot, EZH2 overexpression led to a significant increase in vimentin, while the up-regulation of N-cadherin and the down-regulation of E-cadherin were mild. The protein levels of β-catenin had a slight decrease in EZH2 overexpression cells with no statistical significance. RT-PCR also revealed the dramatic up-regulation of vimentin mRNA and slight changes in other markers (Figure 2D). These results were confirmed by immunofluorescence (Figure 2E). Then, the EZH2 transfected cells were tested.
for cell apoptosis by flow cytometry, but no obvious difference was detected compared with the control group (Figure 2F). These data showed that EZH2 overexpression had more capacity to enhance the migratory and invasive behaviours along with EMT in OSCC cells, while having no evident effects on cell apoptosis.

3.3 | EZH2 knockdown inhibits migration, invasion potential and EMT in OSCC cells

To further investigate the role of EZH2 in the migration, invasion and EMT of OSCC cells, we applied short hairpin RNAs (shRNAs) to knockdown EZH2 expression in Cal-27 and Tca8113 cells, as confirmed by Western blot and RT-PCR (Figure 3A). Wound healing assay showed that EZH2 knockdown of Cal-27 or Tca8113 decreased the migrated area by 31.9% and 23% (Figure 3B). Compared with scrambled control, knockdown of EZH2 impaired cell invasion ability at 34.9% in Cal-27 and 24% in Tca8113 (Figure 3C). Then, we tested EMT markers by Western blot and RT-PCR assay. Results showed that EZH2-knockdown Cal-27 cells exhibited significantly increased epithelial markers E-cadherin and β-catenin, and decreased mesenchymal markers N-cadherin and vimentin, both in protein and mRNA levels (Figure 3D), which were also confirmed by immunofluorescence (Figure 3E). However, no remarkable differences in cell apoptosis between EZH2-knockdown group and scrambled control group were detected (Figure 3F).

3.4 | EZH2 overexpression enhances tumour glycolysis in OSCC cells

Tumour glycolysis has been widely recognized as a central hallmark of human cancer, and emerging studies have shown that glycolysis has close relationship with cancer metastasis.\textsuperscript{12,13,27} To assess the effect of EZH2 on glycolysis of OSCC cells, we detected whether EZH2 could take part in glycolysis using glucose consumption and lactate production assays. As shown in Figure 4A and 4B, the glucose consumption and lactate production levels were significantly increased in EZH2-overexpression cells. However, down-regulation of EZH2 led to lower glucose consumption and lactate production. To determine the correlation of EZH2-induced EMT and tumour glycolysis, OSCC cell lines were pretreated with 2-deoxy-glucose (2-DG), a glycolysis inhibitor. Results showed that 2-DG inhibited EZH2-increased glucose consumption and lactate production levels, and the presence...
of 2-DG suppressed EZH2-induced EMT via down-regulating vimentin and N-cadherin and up-regulating E-cadherin in mRNA levels (Figure 4C). Thus, we concluded that EZH2 overexpression facilitates EMT mediated by OSCC cells glycolysis, which might provide a competitive environment for OSCC cells migration and invasion.

3.5 | EZH2 overexpression elevates the phosphorylation of STAT3 and down-regulates FoxO1

Forkhead box class O1 (FoxO1) is a major modulator of glucose homeostasis and closely correlates with cell glycolysis.28-30 Here, we found that the expression of FoxO1 was negatively regulated by EZH2 in protein and transcription levels (Figure 5A). To further investigate the signalling pathway that mediated FoxO1 expression, we detected the signal transducer and activator of transcription 3 (STAT3) activity in EZH2-overexpressed OSCC cells. STAT3 is a latent transcription factor, which has been researched to disturb FoxO1 transcription.31 Tyrosine-phosphorylated STAT3 at the 705 residue (pY-STAT3) is an active form of STAT3, which can be induced by EZH2. As a classical cancer signalling pathway, EZH2/STAT3 has pivotal roles in cancer growth and metastasis.14,19,32,33 Western blot showed that STAT3 total expression levels remained unchanged in EZH2-overexpressed OSCC cells, whereas pY-STAT3 was enhanced (Figure 5B). To find out whether the effects of EZH2 on FoxO1 expression were mediated by STAT3, we applied shRNAs to knockdown STAT3 expression in OSCC cells, as confirmed by Western blot and RT-PCR. Down-regulation of STAT3 led to increased expression of FoxO1 in both mRNA and protein levels (Figure 5C). Thus, these indicated that EZH2 promoted invasion and glycolysis in human OSCC cells via STAT3 phosphorylation and down-regulation of FoxO1.

3.6 | EZH2 overexpression promotes OSCC tumour growth in vivo

To further illustrate the biological effect of EZH2 in OSCC in vivo, we established the nude mice xenograft model by injecting

![Image](image-url)
EZH2-overexpressed Cal-27 cells and vector control cells subcutaneously. EZH2 overexpression resulted in an increase of bioluminescence from tumours, tumour volume and tumour weight compared with the vector control group, suggesting that EZH2 overexpression promoted OSCC growth in vivo (Figure 6A, 6B and 6C). Tumour specimens were used for RT-PCR to compare the expression levels of EZH2, STAT3 and FoxO1 between two groups. Results confirmed that EZH2 overexpression led to a slight increase in STAT3 mRNA levels and a significant decrease in FoxO1 mRNA levels (Figure 6D).

4 DISCUSSION

Accumulating evidence suggests that tumour glycolysis, known as ‘Warburg effect’ and used by most cancer cells to generate energy for rapid growth and cancer metastasis, exerts great influences on EMT and invasion of various cancers. Here, we revealed that silencing of EZH2 inhibited the tumour glycolysis, EMT, migration and invasion of OSCC cells. EZH2 overexpression increased the tumour glycolysis, EMT, migration and invasion in OSCC cells as well as elevated the phosphorylation of STAT3 and down-regulated FoxO1. FoxO1 expression was enhanced when inhibiting STAT3. In addition, EZH2 expression was positively associated with metastasis and prognosis of OSCC patients. Our data indicated that EZH2 might promote EMT and the metastasis of OSCC through tumour glycolysis, and to our knowledge, this is the first report to reveal the roles of EZH2/STAT3/FoxO1 axis in tumour glycolysis and EMT of OSCC.

Enhancer of zeste homolog 2, a putative candidate oncogene, has been involved in aggressiveness and unfavourable prognosis of most types of cancers. EZH2 could promote lung cancer progression via enhancing VEGF-A/Akt signalling pathway and serve as an independent prognostic factor for lung cancer patients. In ovarian carcinoma cells, EZH2 overexpression could induce TGF-β1 expression and decrease E-cadherin expression and thus support ovarian carcinoma cell invasion. In OSCC tissues, Kidani et al found that EZH2 correlated with clinical stage, lymph node metastasis, tumour size, histological differentiation and higher expression of EZH2 showed poorer prognosis in OSCC, while Shiogama et al reported that aberrant EZH2 was significantly associated with mode of invasion, but not with lymph node metastasis or survival rate. Our data showed that EZH2 overexpression correlated with the metastasis and poor prognosis of OSCC patients. Much should be done in the future. And we found that EZH2 could up-regulate N-cadherin and vimentin, and down-regulate E-cadherin expression both in mRNA and protein levels, thus contributing to OSCC cells migration and invasion, which is in line with the previous publications. This work implied a potentially significant role for EZH2 in promoting EMT and metastasis of OSCC.

Increasing of glycolysis is considered as a common characteristic of cancer cells to rapidly generate energy for EMT and metastasis. For example, glucose restriction in hepatocellular carcinoma cells could down-regulate HSF-1 expression, resulting in direct suppression of Snail 1 expression and up-regulating of E-cadherin expression, thus inhibiting EMT-associated migration and invasion. In uterus endometrial cancer cells, high glucose could promote EMT and lead to a high level of viability and invasion through increasing Glut4 and VEGF/VEGFR expression. However, the effect and mechanism of tumour glycolysis on EMT and metastasis of OSCC is still largely unknown. In this study, we found that EZH2 increased the tumour glycolysis of OSCC cells, thus promoting OSCC metastasis. This was in accordance with the report of Tao and colleagues, who have found that EZH2 overexpression inhibited miR-181b expression, which directly targeted binding site of hexokinase 2 within the 3′-UTR, thus inducing glycolysis and promoting progression in prostate cancer cells. Our results indicated that EZH2 overexpression might manipulate the migrative and invasive abilities through contributing to the tumour glycolysis of OSCC cells.

As a classical cancer pathway, EZH2/STAT3 axis functions as an oncogenic pathway in most cases, where EZH2 enhances STAT3 activity mainly through methylation. For example, EZH2 overexpression, induced by transcriptional regulator NF-YA, could enhance STAT3 activity by mediating its lysine methylation to up-regulate VEGF expression and promote angiogenesis in melanoma cells. Moreover, EZH2 could also promote phosphorylation of pY705 on STAT3 via other mechanisms independent of STAT3 methylation. Venkatesan and colleagues demonstrated that EZH2 interaction with VAV family proteins, which were involved in migration and proliferation of cancer cells, contributed to initial neoplastic transformation via activating STAT3 pathway. In this study, through Western blot analysis, we found that up-regulation of EZH2 could significantly enhance the expression of tyrosine-phosphorylated STAT3 at the 705 residue, while total STAT3 expression remained unchanged. Consistent with major roles of EZH2, we suggested that EZH2 might regulate invasion and tumour glycolysis of OSCC cells through activating STAT3. However, Ozes et al reported that phosphorylation of EZH2 at threonine 372 could inhibit ovarian carcinoma cells proliferation and migration in vitro and decrease ovarian xenograft tumour growth in vivo, through binding to STAT3 and reducing levels of pSTAT3, which might be due to the differences in cancer microenvironment.

As a critical downstream protein in STAT3 signalling, FoxO1 is a key regulator of glucose homeostasis, cell proliferation and apoptosis. Studies have shown that FoxO1/3/4 knockout mice had obvious changes in glucose metabolism than wild-type mice and had decreased gluconeogenesis and increased glycolysis. In epithelial cells, FoxO1 could act as a gatekeeper of endothelial quiescence, which decelerated metabolic activity by reducing glycolysis and mitochondrial respiration. Moreover, FoxO1 could up-regulate PDK4 expression and impair glucose oxidation, thus leading to right ventricular hypertrophy. Besides, being an important glucose metabolism-related protein, FoxO1 is associated with EMT of cancer. In metastatic bladder cancer cells, FoxO1 was down-regulated by miR-145 and down-regulation of FoxO1 promoted cancer cell growth and invasion. Evidence also showed that FoxO1 expression could be transcriptionally decreased by activating STAT3 signalling. And in
breast cancer cell lines, combination therapy of trastuzumab and MPA markedly decreased constitutive activation of STAT3 and resulted in higher expression of proapoptotic factors such as p27 and FoxO1. Hence, we showed that overexpression of EZH2 decreased levels of FoxO1 transcriptionally and translationally and down-regulation of STAT3 led to increased expression of FoxO1 in both mRNA and protein levels. These results confirmed that EZH2 contributed to OSCC cell invasion and tumour glycolysis through regulating STAT3/ FoxO1 axis. While in highly aggressive prostate cancer, FoxO1 could bind to Twist1 promoter, induce EMT and invasion via AKT/FoxO1/ Twist1 pathway. This discrepancy might be due to different roles which FoxO1 played in the chosen tumours. Much should be done in the future.

In summary, we described a novel mechanism of EZH2/STAT3/ FoxO1 axis in tumour glycolysis-related EMT and invasion of OSCC cells. We also provided evidence for oncogenic roles of EZH2 in OSCC. These results indicated that EZH2 might act as a potential biomarker for OSCC diagnosis and treatment.

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CONFLICTS OF INTEREST
The authors report no conflicts of interest in this work.

AUTHORS’ CONTRIBUTIONS
MZ, MXC, YLT and XHL designed the research study. MZ, MXC and LL conducted the cell experiments. MZ, MXC and KW conducted the animal model experiments. SSW, HFW and YJT conducted the experiments on the human samples. MZ, MXC, YLT and XHL were responsible for writing of manuscript. MXC, SSW and XJL revised the manuscript. All authors reviewed the manuscript.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of the article.

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