Zinc finger E-box binding homeobox 2 functions as an oncogene in human laryngeal squamous cell carcinoma

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Abstract. Zinc finger E-box binding homeobox 2 (ZEB2) is a member of the Zfh1 family of two-handed zinc finger/homeodomain proteins. To date, the role of ZEB2 in human laryngeal carcinoma has not been clearly defined. In the present study, the level of ZEB2 expression in laryngeal squamous cell carcinoma (LSCC) tissues and adjacent normal tissues was evaluated using reverse transcription-quantitative polymerase chain reaction. The effects of ZEB2 on the growth, migration, invasion, cell cycle distribution and apoptosis of laryngeal cancer cells were also explored using MTT, Transwell and flow cytometry assays. It was identified that ZEB2 was upregulated in LSCC tissues compared with normal tissues. Silencing of ZEB2 inhibited the viability, migration and invasion of LSCC cells. It was also observed that ZEB2 silencing induced cell cycle arrest and apoptosis in LSCC cells. Furthermore, ZEB2 silencing inhibited the process of epithelial-mesenchymal transition. Overall, the results indicated that ZEB2 promotes the progression of LSCC and that it may be a potential target for the treatment of this type of cancer.

Introduction

Laryngeal cancer is one of the most commonly observed malignancies of the head and neck, and accounts for ~25% of tumors in this region (1). Laryngeal cancer accounts for ~1.1% of all newly diagnosed cancer cases and for nearly 1% of all tumor-associated mortalities worldwide (2). Among different subtypes, laryngeal squamous cell carcinoma (LSCC) accounts for >90% of laryngeal cancer cases, and is more prevalent in middle-aged and elderly males (3). Although advances have been achieved in the management of this tumor, the survival rates of patients with LSCC have remained unfavorable for the past 30 years (4). Therefore, it is urgent to unveil the molecular mechanisms underlying the development of LSCC.

Zinc finger E-box binding homeobox 2 (ZEB2), a transcription factor that belongs to the Zfh1 family, has been found to be overexpressed in various types of cancer, including colorectal, prostate and pancreatic cancer (5-7). Mounting evidence has indicated that ZEB2 is involved in the initiation and development of malignancies (8). Furthermore, it has been reported that ZEB2 promotes the epithelial-mesenchymal transition (EMT), a pathological process leading to specific morphological and phenotypic alterations during cancer metastasis in tumor cells (9,10). According to previous studies, ZEB2 was significantly upregulated in LSCC (11,12). However, the function of ZEB2 in LSCC remains largely unknown.

In the present study, the expression of ZEB2 in LSCC and adjacent normal tissues was investigated. In addition, the effect of ZEB2 silencing on the proliferation, migration, invasion, cell cycle distribution, apoptosis and EMT of LSCC cells was examined. The results revealed that ZEB2 functioned as an oncogene in LSCC and that targeting ZEB2 may be a promising therapeutic target in the management of LSCC.

Patients and methods

Patients and specimens. Between January 2014 and February 2015, laryngeal cancer tissues (n=46) and the corresponding adjacent non-tumor tissues (n=46) were collected from laryngeal cancer patients in the Department of Otorhinolaryngology, Head and Neck Surgery (Lihuili Hospital, Ningbo, China). Patient characteristics are presented in Table I. There were 21 female and 25 male patients, aged from 40-79 and the mean age was 59. All patients were diagnosed with LSCC according to WHO criteria (13). The research was approved by the Institutional Review Board of Lihuili Hospital,

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and written informed consent was obtained from each patient prior to surgery.

Cell culture. Human laryngeal cancer AMC-HN8 cells (cat no. 0143321) were obtained from the Shanghai Cell Bank of the Chinese Academy of Science (Shanghai, China). Cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37˚C with 5% CO_2.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the cells or tissues by TRIzol reagent (Thermo Fisher Scientific, Inc.). The quality and concentration of RNA was measured by NanoDrop (Thermo Fisher Scientific, Inc.). RNA samples were reverse transcribed using cDNA synthesis kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. qPCR was then performed using the SYBR ExScript qRT-PCR kit (Takara Biotechnology Co., Ltd., Dalian, China) and conducted on an Applied Biosystems 7500 Real-Time PCR system (Thermo Fisher Scientific, Inc.). Conditions of the PCR amplification were: 94˚C (5 min), then 30 cycles at 94˚C (30 sec), 60˚C (45 sec) and 72 C (45 sec) with a final extension at 72 C for 10 min.

The primer pairs used in qPCR were as follows: ZEB2 forward, 5'-AAGGAGCAGGTAATCGCAAG-3', and reverse, 5'-GGAACCAGAATGGGAGAAACG-3'; GAPDH forward, 5'-AGAAGGCTGGGGCTCATTTG-3' and reverse, 5'-AGGGGCCATCCACAGTCTTC-3'. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous control. The expression fold changes were calculated using the $2^{-\Delta\Delta Cq}$ method (14).

Silencing of ZEB2. ZEB2 was silenced in AMC-HN8 cells with two different small interfering RNAs (siRNAs), which were synthesized by GeneChem Co., Ltd. (Shanghai, China). The siRNA sequences were as follows: si-ZEB2-1, 5'-TGAAAACATAGTCCCCAACA-3'; and si-ZEB2-2, 5'-CTGGACAACAAAAGCACT-3'. Briefly, cells were seeded at the density of $1x10^6$ cells/well in 6-well plates and incubated until 70% confluency was reached. On the following day, the appropriate amount of siRNA against ZEB2 (si-ZEB2-1 and si-ZEB2-2) or negative control siRNA (si-NC) were transfected into the cells using Lipofectamine 3000 reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

Cell proliferation assay. Cell viability was evaluated by an MTT assay. Briefly, cells were seeded in a 96-well plate ($5x10^3$ cells/well) and then treated for 24 h with or without siRNAs. A total of 50 μl MTT solution (5 mg/ml) was added to each well, and the cells were incubated for 4 h. The medium was then removed, and 200 μl dimethyl sulfoxide (DMSO) was added to each well. Subsequently, the absorbance of the solutions was measured on a BioTek microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) at 595 nm. The relative cell viability (%) was measured by comparing the absorbance at 595 nm with that of the vehicle control, which was treated with 0.1% DMSO.

Cell cycle distribution analysis. Cells grown in 6-well plates ($1x10^6$ cells/well) were treated with siRNA against ZEB2 or si-NC. The floating cells were discarded, and attached cells were harvested and fixed in ice-cold 70% ethanol. Next, the fixed cells were washed with phosphate-buffered saline (PBS) and resuspended in PBS containing RNase A (20 μg/ml). The cells were fixed with 70% ethanol and stained with propidium iodide (PI), and the cell cycle distribution was evaluated using FACSVerse™ flow cytometer (Beckman Coulter, Inc., Fullerton, CA, USA). Data were analyzed using FlowJo version 10 software (FlowJo LLC, OH, USA).

Apoptosis assay. Cells grown in 6-well plates were treated with siRNA against ZEB2 or si-NC. All the cells were harvested and fixed in ice-cold 70% ethanol. The fixed cells were then washed with PBS, resuspended in PBS containing RNase A (20 μg/ml) and stained with the Annexin V-FITC/PI Apoptosis Detection kit (BD Biosciences, Franklin Lakes, NJ, USA). Cell apoptosis was evaluated using FACSVerse™ flow cytometer, and data were analyzed using FlowJo version 10 software.

Cell migration and invasion assays. For the migration assays, at 48 h post-transfection, $5x10^4$ cells in serum-free medium were placed into the upper chamber of a Transwell insert (24-wells plate, 8-μm pore size; EMD Millipore, Billerica, MA, USA). For the invasion assays, at 48 h post-transfection, $1x10^5$ cells in serum-free medium were placed into the upper chamber of a Transwell insert coated with Matrigel (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Medium containing 10% FBS was added to the lower chamber for both assays. Subsequent to incubation for 24 h, the cells remaining on the upper membrane were removed with cotton wool. Cells that had migrated or invaded through the membrane were stained with methanol and 0.1% crystal violet, and counted under an inverted microscope (Olympus Corporation, Tokyo, Japan). Experiments were independently repeated three times.

Western blot assay. Following transfection, cells were lysed with radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Shanghai, China). The concentration of protein lysates were assayed by the Bradford kit (Beyotime Institute of Biotechnology, Shanghai, China). The lysates (40 μg) were resolved by 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 10% skimmed-milk at room temperature for 1 h. Subsequently, the membranes were incubated overnight at 4˚C with primary antibodies against the following: ZEB2 (23312), obtained from Wuhan Sanying Biotechnology (Wuhan, China); matrix metalloproteinase MMP-2 (ab37150), MMP-9 (ab58803), E2 promoter binding factor 1 (E2F1) (ab179445) and vimentin (ab137321), purchased from Abcam (Cambridge, MA, USA); p21 (2947), cyclin D1 (2922), cyclin-dependent kinase (CDK)4 (12790), CDK6 (3136), caspase-3 (9662), caspase-8 (9746), caspase-9 (9502), cleaved poly(ADP-ribose) polymerase (PARP) (9541), B-cell lymphoma 2 (Bcl-2) (4223) and Bcl-2-associated X protein (Bax) (2774), all purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA); E-cadherin (743712) and N-cadherin (746629), from BD Biosciences; and GAPDH (G5262), obtained from Sigma-Aldrich (Merck KGaA). The samples were then incubated with the appropriate secondary

Table I. Clinical characteristics of 46 laryngeal squamous cell carcinoma patients.

Specimen no.	Histologic differentiation	Age (years)	TNM stage
1	Poor	76	T1N0M0
2	Moderate	53	T4N0M0
3	Moderate	64	T1N0M0
4	Moderate	49	T1N0M0
5	Poor	64	T4N2M0
6	Good	57	T3N0M0
7	Poor	57	T4N0M0
8	Moderate	64	T1N0M0
9	Moderate	57	T4N2M0
10	Poor	61	T4N1M0
11	Poor	56	T3N2M0
12	Good	70	T3N0M0
13	Good	51	T4N1M0
14	Moderate	59	T4N1M0
15	Poor	68	T2N1M0
16	Poor	79	T1N0M0
17	Poor	55	T4N1M0
18	Good	48	T4N2M0
19	Moderate	59	T3N2bM0
20	Poor	67	T1N0M0
21	Poor	59	T2N1M0
22	Moderate	64	T1N0M0
23	Moderate	57	T1N0M0
24	Good	52	T1N0M0
25	Poor	48	T1N0M0
26	Poor	60	T1N0M0
27	Moderate	54	T1N0M0
28	Good	40	T2N0M0
29	Good	45	T2N0M0
30	Moderate	63	T3N2bM0
31	Poor	51	T1N0M0
32	Poor	62	T2N2MO
33	Good	51	T3N1M0
34	Moderate	59	T4N1M0
35	Good	63	T2N2M0
36	Moderate	74	T3N1M0
37	Poor	52	T1N0M0
38	Poor	72	T4N2M0
39	Moderate	63	T2N0M0
40	Moderate	60	T1N0M0
41	Good	51	T4N0M0
42	Good	51	T3N1M0
43	Moderate	55	T1N0M0
44	Moderate	54	T1N0M0
45	Poor	66	T1N0M0
46	Poor	54	T1N0M0

antibodies at room temperature for 1 h (Sigma-Aldrich; Merck KGaA). Signals were visualized using an enhanced chemiluminescence reagent (Pierce; Thermo Fisher Scientific, Inc.).

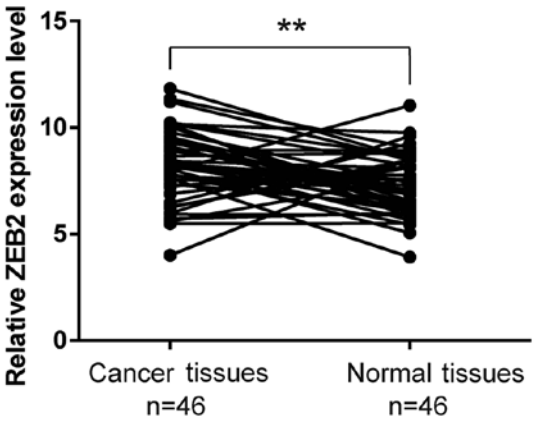

Figure 1. ZEB2 was significantly upregulated in laryngeal cancer tissues. ZEB2 mRNA levels were examined in laryngeal cancer tissues (n=46) and the corresponding non-tumor tissues by reverse transcription-quantitative polymerase chain reaction, and the results were normalized to GAPDH expression. P-values between samples were calculated by performing a t-test. **P<0.01. ZEB2, zinc finger E-box binding homeobox 2.

Statistical analyses. All the experiments were conducted at least three times. The data were analyzed using GraphPad Prism version 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA). The results are presented as the mean ± standard deviation, and the differences between two groups or multiple groups were measured using Student's t-test or using analysis of variance followed by Tukey's test, respectively. P<0.05 was considered to indicate a statistically significant difference.

Results

ZEB2 is significantly upregulated in laryngeal cancer tissues. ZEB2 expression levels were investigated using RT-qPCR assays in 46 laryngeal cancer tissues and adjacent histologically normal tissues. The results demonstrated that ZEB2 expression was significantly higher in tumor tissues compared with that in adjacent normal tissues (P<0.01; Fig. 1).

Silencing of ZEB2 represses laryngeal cancer cell proliferation, migration and invasion in vitro. To investigate the potential role of ZEB2 in laryngeal cancer cell proliferation, two siRNAs against ZEB2 were transfected into laryngeal cancer AMC-HN8 cells. The RT-qPCR and western blot analyses confirmed that the expression of ZEB2 was effectively reduced following transfection (Fig. 2A). The MTT assay demonstrated that the proliferation of AMC-HN8 cells was significantly suppressed by ZEB2 downregulation, as compared with that in the si-NC group (Fig. 2B). The role of ZEB2 in cell migration and invasion was then further evaluated. As shown in Fig. 2C and D, ZEB2 silencing resulted in marked repression of the migration and invasion abilities of AMC-HN8 cells (P<0.01). It was also observed that the protein expression levels of MMP-2 and MMP-9 were downregulated following silencing of ZEB2 (Fig. 2E).

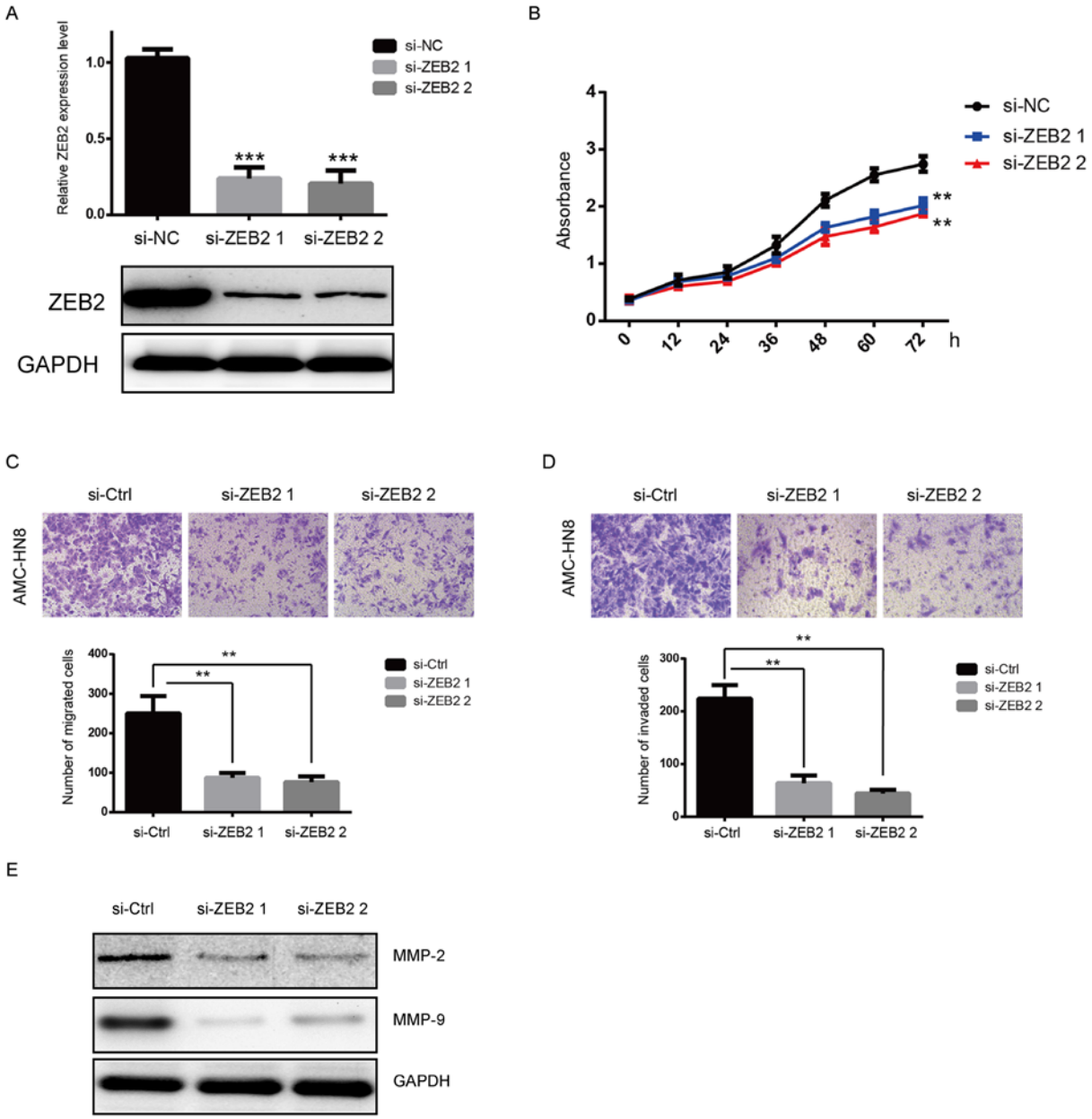

Figure 2. ZEB2 promotes the cellular proliferation, migration and invasion of laryngeal cancer cells. (A) ZEB2 expression levels in AMC-HN8 cells transfected with si-NC and two si-ZEB2 (si-ZEB2-1 and si-ZEB2-2) were detected by reverse transcription-quantitative polymerase chain reaction (top) and western blot assay (bottom). (B) The proliferation of AMC-HN8 cells transfected with si-NC or si-ZEB2 was determined at the indicated time points by MTT assay. (C) Migration and (D) invasion assays were performed in Transwell chambers without or with Matrigel pre-coating, respectively, following cell transfection with si-NC and si-ZEB2. (E) Levels of key proteins in metastasis, as determined by western blot assay. **P<0.01 and ***P<0.001, vs. si-NC group. ZEB2, zinc finger E-box binding homeobox 2; si-, small interfering RNA; NC, negative control.

Downregulation of ZEB2 induces cell cycle arrest in laryngeal cancer cells. Furthermore, the role of ZEB2 in the cell cycle distribution was investigated in AMC-HN8 cells using flow cytometry analysis. As shown in Fig. 3A and B, ZEB2 silencing caused cell cycle arrest at the G_0/G_1 phase. The percentage of cells in the G_0/G_1 phase significantly increased from 42.7% in the control group to 64.5 and 66.3% in cells with si-ZEB1 and si-ZEB2, respectively (P<0.01). Simultaneously, the proportion of cells in S phase markedly decreased (Fig. 3B). To examine the molecular mechanism responsible for the cell cycle arrest in G_0/G_1 phase subsequent to ZEB2 downregulation, the expression levels of cell cycle regulatory proteins were determined. It was observed that the expression levels of cyclin D1, CDK4, CDK6 and E2F1 proteins were notably reduced, while p21 levels evidently increased following the downregulation of ZEB2. All these data suggested that ZEB2 silencing induced a G_0/G_1 block in laryngeal cancer cells via altering the expression of key proteins in the cyclin-CDK/E2F1 signaling pathway.

Downregulation of ZEB2 causes apoptosis in laryngeal cancer cells via the intrinsic pathway. The study further investigated whether ZEB2 downregulation affected cell apoptosis using an Annexin V-FITC/PI apoptosis detection kit. As shown in Fig. 4A and B, flow cytometry analysis revealed that ZEB2 silencing significantly promoted the apoptosis of AMC-HN8 cells. Cell apoptosis mainly proceeds through two pathways,

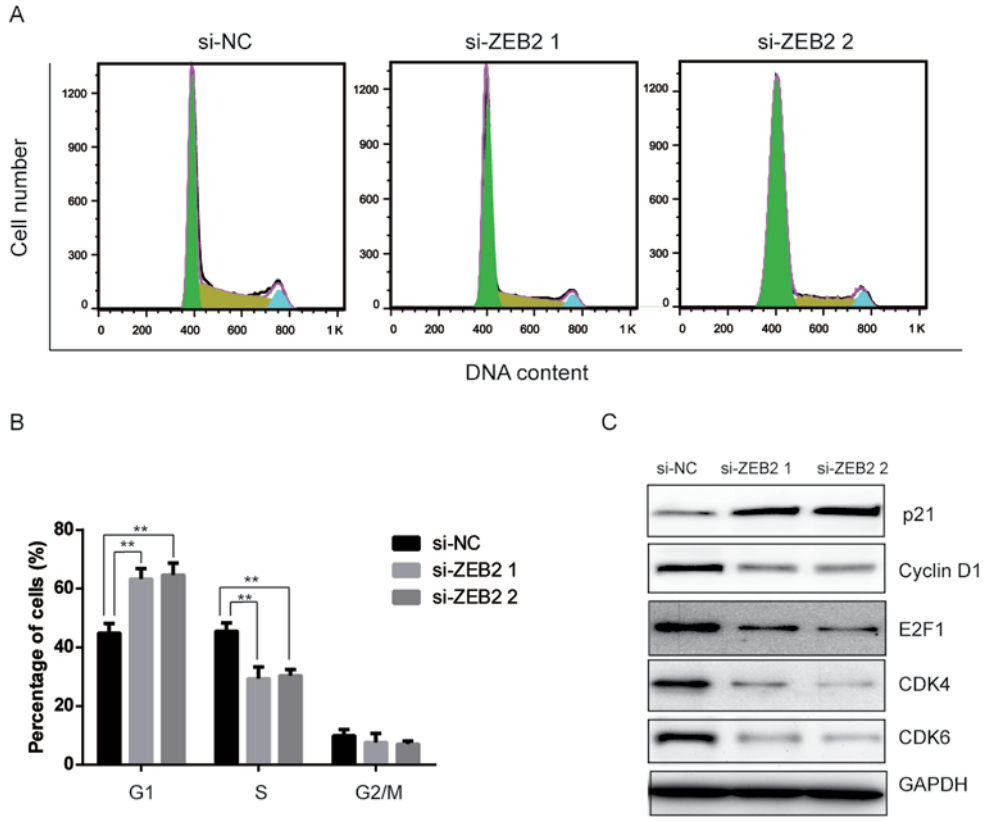

Figure 3. Downregulation of ZEB2 induced cell cycle arrest in laryngeal cancer cells. AMC-HN8 cells were transfected with si-NC or si-ZEB2 for 48 h, and then cells were collected. (A) Cell cycle distribution was analyzed using propidium iodide staining and flow cytometry. (B) Quantitative analysis of cell cycle distribution. (C) Expression of cell cycle-associated proteins, including p21, cyclin D1, E2F1, CDK4 and CDK6, were determined by western blot assay. **P<0.01. ZEB2, zinc finger E-box binding homeobox 2; si-, small interfering RNA; NC, negative control; E2F1, E2 promoter binding factor 1; CDK, cyclin-dependent kinase.

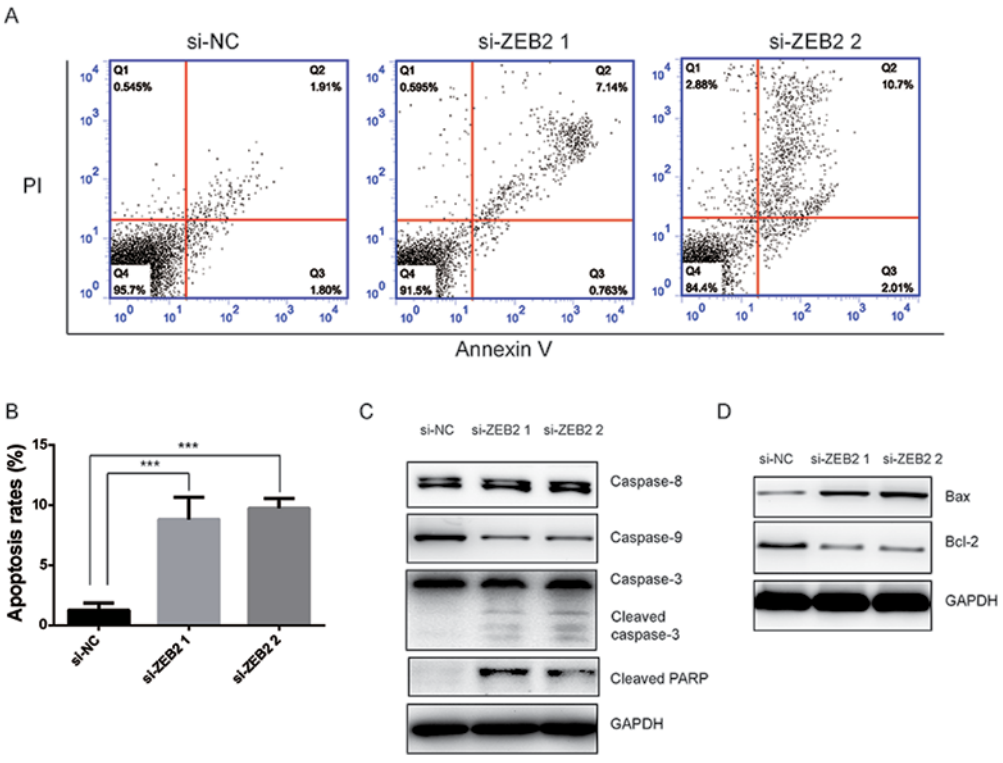

Figure 4. Downregulation of ZEB2 induced laryngeal cancer cell apoptosis through the intrinsic pathway *in vitro*. (A) Flow cytometry was applied to analyze the apoptosis of AMC-HN8 cells transfected with si-NC or si-ZEB2 for 48 h. (B) Quantitative analysis of cell apoptosis. Western blot assay was used to determine the levels of (C) caspase-3/8/9 and PARP, and (D) Bcl-2 and Bax proteins in AMC-HN8 cells transfected with si-NC or si-ZEB2. ***P<0.001. ZEB2, zinc finger E-box binding homeobox 2; si-, small interfering RNA; NC, negative control; PARP, poly(ADP-ribose) polymerase; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein.

including the extrinsic pathway, which is initiated by caspase-8, and the intrinsic pathway, which is initiated by caspase-9 (15). To further explore the potential role of ZEB2 in apoptosis, western blot analysis was performed to detect the levels of associated proteins. Upon silencing of ZEB2, downregulation of caspase-9 and cleavage of caspase-3 were observed, and cleavage of PARP levels were evidently increased, while the level of caspase-8 was not affected (Fig. 4C). Furthermore, the protein expression levels of Bcl-2 and Bax, which are key proteins that regulate the intrinsic pathway, were respectively downregulated and upregulated following ZEB2 downregulation. These data implied that the intrinsic pathway was activated following the silencing of ZEB2.

ZEB2 affects the levels of E-cadherin proteins. The EMT has been reported to be involved in cancer invasion and metastasis (16). Therefore, the present study examined whether ZEB2 was able to affect the process of EMT in AMC-HN8 cells. Western blot analysis was performed to detect the expression levels of EMT markers, including E-cadherin, N-cadherin and vimentin. As indicated in Fig. 5, downregulation of ZEB2 notably induced E-cadherin expression, but repressed the expression of N-cadherin and vimentin proteins. These results indicated that silencing of ZEB2 inhibited the process of EMT in AMC-HN8 cells.

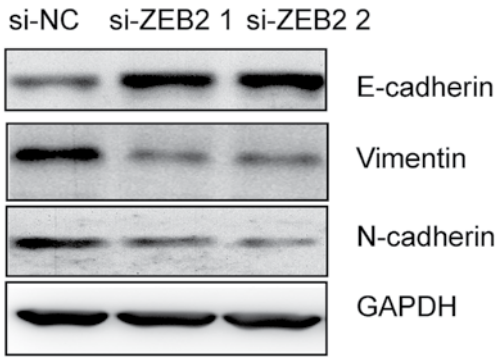

Figure 5. Downregulation of ZEB2 reverses the epithelial-mesenchymal transition in AMC-HN8 cells. Western blot assay was used to determine the expression of key EMT-associated proteins in AMC-HN8 cells transfected with si-NC or si-ZEB2 for 48 h. ZEB2, zinc finger E-box binding homeobox 2; si-, small interfering RNA; NC, negative control.

Discussion

Although several therapeutic approaches have been developed, the overall 5-year survival rates for patients with LSCC remain unsatisfactory, largely due to metastasis and recurrence (3). A better understanding of the molecular mechanisms underlying the proliferation, differentiation and progression of LSCC is vital for the development of efficient therapeutic strategies. In the present study, the role of ZEB2 in LSCC was investigated, and the data provide a mechanistic basis for targeting ZEB2 in patients with LSCC.

ZEB2, a transcription factor that belongs to the ZEB family, has been reported to be involved in the development of various human malignancies. Knockdown of its expression may repress the proliferation and progression of these tumors. For instance, a recent study revealed that ZEB2 promoted tumor metastasis and was correlated with poor prognosis in human colorectal cancer (17). In hepatocellular carcinoma, ZEB2 was reported to promote tumor growth and metastasis (18). In gastric cancer, ZEB2 may affect the cellular response to cisplatin (19). However, few studies have examined the roles of ZEB2 in LSCC. The present study demonstrated that ZEB2 was highly expressed in LSCC tissues as compared with adjacent normal tissues. It was also observed that silencing of ZEB2 effectively reduced the proliferation, migration and invasion of LSCC cells.

Mounting evidence has revealed the vital role of the MMP family in cancer metastasis (20). Among the MMP family members, MMP-2 and MMP-9 stand out for their ability to degrade collagen IV, which is the major extracellular component of the basement membrane (21). The overexpression of MMP-2/-9 is associated with an aggressive malignant phenotype and adverse prognosis in patients with cancer (22). In the current study, ZEB2 silencing resulted in decreased MMP-2/-9 levels, which provided an insight into the investigation of ZEB2 on LSCC metastasis. These findings were similar to those of previous studies, which indicated that high ZEB2 expression levels may be positively associated with worse tumor biological features (17,18).

The present study also demonstrated an increase in the percentage of LSCC cells at the G1 phase following ZEB2 silencing. Cell cycle progression is regulated by a cyclin kinase inhibitor and the cell cycle regulators p21 and p27, which are upregulated during cell cycle arrest (23). CDKs are activated via binding to cyclin complexes, and their activity can be repressed by cyclin kinase inhibitors (24). When cell cycle arrest occurs, CDKs and cyclin complexes can be repressed by the expression of CDK inhibitors (24). The data reported in the present study revealed that downregulation of ZEB2 led to upregulation of p21 and downregulation of cyclin D1, E2F1, CDK4 and CDK6. Taken together, these findings are in line with a previous study, which also reported that downregulation of ZEB2 causes cell cycle arrest at the G1 phase in glioma cells (25).

Silencing of ZEB2 also resulted in a significant increase in the apoptosis in LSCC cells. There are mainly two pathways that lead to apoptosis, namely the extrinsic and intrinsic pathways, which are initiated by caspase-8 and caspase-9, respectively (26). During apoptosis, activation of caspase-8 and caspase-9 can both lead to the activation of caspase-3. Following ZEB2 silencing, caspase-9 and caspase-3, but not caspase-8, were activated. Furthermore, the intrinsic apoptotic pathway is known to be regulated by the Bcl-2 family members (27). In the current study, the pro-apoptotic Bax was upregulated and the anti-apoptotic Bcl-2 protein was downregulated following ZEB2 silencing. These findings indicated that ZEB2 affects apoptosis via the intrinsic apoptotic pathway in LSCC cells. Notably, although apoptosis is observed, few cells were detected at the sub-G1 phase. This discrepancy may be due to technical reasons, since the floating cells were discarded when the cell cycle distribution was analyzed, whereas all cells were collected in order to measure the apoptosis.

EMT is a biological process where epithelial cells lose their polarity and undergo transition into a mesenchymal phenotype (28). The occurrence of EMT is accompanied by downregulation of the epithelial marker protein E-cadherin,

whereas mesenchymal marker proteins, such as N-cadherin and vimentin, are upregulated. Accumulating evidence indicates that ZEB2 can trigger EMT, thereby increasing cancer metastasis (8,29,30). Therefore, the present study investigated whether ZEB2 was able to affect the EMT in LSCC cells. The results revealed higher expression levels of E-cadherin, and lower expression levels of N-cadherin and vimentin in the cells with downregulation of ZEB2. These findings indicated that ZEB2 promoted the EMT in LSCC cells.

In conclusion, the current study investigated the level of ZEB2 mRNA expression in LSCC tissues and found it was upregulated compared to the normal tissues. Then we investigated the function of ZEB2 *in vitro*. Downregulation of ZEB2 inhibited the proliferation, migration, invasion, cell cycle progression, apoptosis and EMT of LSCC cells. Taken together, the results provide new evidence that ZEB2 may represent a novel therapeutic target for the treatment of LSCC. However, the limitations of the present study included the fact that no studies have yet been conducted in animal models to confirm the *in vitro* findings. Other limitations include the failure to demonstrate the specific mechanism by which ZEB2 affects the processes described herein; therefore, further research is required.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QL and LM performed the experiments. ZW, GW and QH analyzed the data. ZS and RY designed the experiments and drafted the manuscript.

Ethics approval and consent to participate

This project was approved by the Ethics Committee at the Ningbo University, and all patients gave informed consent.

Patients' consent for publication

All patients agreed to publication.

Competing interests

The authors declare that they have no competing interests.

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