Original Article
OVOL1 inhibits oral squamous cell carcinoma growth and metastasis by suppressing zinc finger E-box binding homeobox 1

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Abstract: Introduction: Lymph node metastasis is the primary cause of death in oral squamous-cell carcinoma (OSCC) patients, so understanding the underlying molecular mechanism is critical for treating metastatic OSCC. OVOL1, a transcription factor, functions as a "break" to repress metastasis in breast cancer and prostate cancer. Aims: To explore the roles of OVOL1 in the progression of OSCC, especially during metastasis. Results: The OVOL1 level was increased significantly in non-metastatic OSCC tissues and negatively correlated with ZEB1 level. OVOL1 repressed ZEB1 expression by directly binding to the promoter region of ZEB1. OVOL1 functioned as a tumor suppressor, and suppressed SCC-152 cells proliferation, migration, and invasion and promoted apoptosis. ZEB1 almost fully rescued the overexpressed OVOL1 function in SCC-152 cells. Conclusion: OVOL1 overexpression contributes to the progression of OSCC through inhibiting ZEB1, which may provide a marker for prognosis in OSCC.

Keywords: Oral squamous cell carcinoma, OVOL1, ZEB1, metastasis

Introduction

Oral squamous cell carcinoma (OSCC), a malignant neoplasm of stratified squamous epithelium in the oral cavity, is the most common type of oral cancer, accounting for more than 90% of all oral cancers and 38% of head and neck tumors [1]. Although surgery and chemotherapy have improved over the past decades, oral cancer is still closely related to poor prognosis and low survival rate [2]. Patients with tumor at an advanced stage usually suffer from tumor cell invasion to surrounding tissues, and have an extremely high risk of second malignancy during the patient's lifetime [3]. Therefore, it is important to understanding the mechanisms driving OSCC progression and metastasis.

Epithelial to mesenchymal transition (EMT) allows cancer cells to dedifferentiate and acquire enhanced migratory and invasive properties which play an important role during tumor metastasis [4]. Hence, deciphering the molecular mechanisms that maintain the hybrid epithelial/mesenchymal phenotype may provide a new choice for anti-metastasis strategies.

OVO-like proteins (OVOL) are a group of zinc finger protein family transcription factors that regulate gene expression in various differentiation processes [5]. Increasing evidence indicates that OVOL genes, especially OVOL1 and OVOL2, are involved in EMT in human cancer including breast cancer and prostate cancer [6]. It is known that OVOL proteins may function as an EMT "break" through repressing ZEB1 and inducing miR-200 family members. However, the role of OVOL proteins during carcinogenesis of OSCC is still unknown.

In this study, we analyzed the expression of OVOL1, OVOL2, and ZEB1 in metastatic and non-metastatic OSCC tissues. The regulatory relationships between OVOL1 and ZEB1 were examined in OSCC cells.

Materials and methods

Patients

Fifty patients with OSCC (25 non-metastasis and 25 lymph node metastasis) were recruited for OVLO1, and OVLO2 levels detection. The

Table 1. Clinical characteristics of patients with OSCC in this study

Patient	Site	Gender	Age (yr)	Pathologic stage	HSV infection
P0032	OC-T	M	63	T1N0M0	HSV1
P0052	OC-T	M	49	T2N0M0	Negative
P0043	OC-FOM	M	55	T4N0M0	Negative
P0106	OC-T	F	53	T1N0M0	Negative
P0112	OC-FOM	M	69	T2N0M0	HSV1
P0156	OC-FOM	M	63	T1N0M0	Negative
P0193	OC-T	M	57	T2N0M0	Negative
P0266	OC-T	M	44	T2N0M0	Negative
P0372	OC-T	M	57	T1N0M0	Negative
P0392	OC-T	M	61	T3N0M0	HSV1
P0397	OC-FOM	M	66	T1N0M0	Negative
P0432	OC-FOM	M	55	T3N0M0	HSV2
P0439	OC-FOM	M	58	T2N0M0	Negative
P0444	OC-T	M	60	T2N0M0	Negative
P0531	OC-T	M	54	T4N0M0	Negative
P0536	OC-T	M	62	T2N0M0	Negative
P0621	OC-FOM	M	58	T1N0M0	HSV1
P0775	OC-T	F	71	T2N0M0	HSV1
P0812	OC-T	F	68	T2N0M0	Negative
P0843	OC-T	M	63	T3N0M0	Negative
P0915	OC-FOM	M	66	T1N0M0	Negative
P0953	OC-T	F	54	T3N0M0	Negative
P1016	OC-FOM	M	59	T3N0M0	Negative
P1083	OC-T	M	63	T2N0M0	HSV1
P1106	OC-T	M	63	T1N0M0	Negative
P0008	OC-FOM	M	55	T4N1M1	Negative
P0011	OC-FOM	M	58	T3N1M0	Negative
P0018	OC-T	M	62	T2N1M1	Negative
P0028	OC-T	M	55	T3N2M0	Negative
P0037	OC-T	F	66	T2N2M1	HSV1
P0055	OC-T	M	69	T3N1M0	Negative
P0059	OC-FOM	M	56	T4N3M1	Negative
P0064	OC-FOM	M	58	T3N1M1	HSV1
P0066	OC-FOM	M	61	T2N1M0	Negative
P0087	OC-T	F	57	T3N2M1	Negative
P0091	OC-T	F	60	T3N3M1	Negative
P0094	OC-T	M	62	T2N1M0	HSV2
P0119	OC-T	M	59	T2N2M0	Negative
P0143	OC-FOM	F	70	T4N3M1	Negative
P0195	OC-T	M	68	T3N2M1	Negative
P0229	OC-T	M	59	T3N1M0	Negative
P0398	OC-FOM	M	62	T2N1M0	Negative
P0463	OC-T	M	55	T2N2M1	Negative
P0555	OC-FOM	M	47	T1N3M1	HSV2
P0588	OC-T	M	52	T2N1M0	Negative

clinical characteristics are shown (**Table 1**). Primary tumor tissue samples were collected from patients undergoing surgical resection of primary tumor at Affiliated Hospital of Stomatology, Nanjing Medical University. Written informed consents were obtained from all patients and the research protocol was approved by review boards and by the Ethics Committee in Research of Affiliated Hospital of Stomatology, Nanjing Medical University. Tumor samples were snap-frozen in liquid nitrogen immediately after surgery. Analysis of hematoxylin and eosin-stained sections by the study pathologists confirmed at least 70% of tumor cells in all OSCC samples.

DNA extraction and PCR amplification

50 micron sections were cut from each tissue specimen block and DNA was extracted using RNAprep pure Tissue Kit (TIANGEN BioTec, Beijing, China). PCR was performed by processing the extracted DNA with primers that are specific for HSV-1 and HSV-2 genes. The primers used for HSV1 are Forward: GTTAGGGAGTTGT-TCGGTCATAAGCT, Reverse: TCGGCCATCT-TGAGCATCC; the HSV2 primers are: Forward: GTCGGTGTGGTGTTCGGTCATAAGCT, Reverse: GGCTGAATCTGGTAAACACGCTTC.

Cell culture

Oral squamous cell carcinoma (OSCC) SCC-152 cells were obtained from the American Type Culture Collection (ATCC) and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA). Human embryonic kidney 293T cells were cultured in DMEM medium containing 10% fetal bovine serum (Hyclone, Logan, UT, USA), 100 IU/ml penicillin and 10 mg/mL streptomycin. All cells were cultured at 37°C under an atmosphere containing 5% CO_2.

Western blotting

To examine the level of target genes, protein was extracted from tissues or cells and then boiled in SDS/β-mercaptoethanol sample buffer. 20 μg protein samples were loaded into each lane of 10% polyacrylamide gels. After electrophoresis, the

P0667	OC-T	M	49	T1N2M0	Negative
P0687	OC-T	M	64	T2N2M0	HSV1
P0844	OC-FOM	M	63	T4N2M1	HSV1
P0933	OC-T	M	65	T3N1M0	Negative
P1089	OC-T	M	59	T3N2M1	HSV1

OC-T: Oral-Cavity - Tongue, OC-FOM: Oral Cavity - Floor of the Mouth. Pathological Stage describes the metastatic status of the samples, with N0 meaning no lymph node metastasis at the time of diagnosis and N>0 indicating the presence of lymph node metastasis.

proteins in the gels were blotted onto PVDF membranes by electrophoretic transfer. Rabbit anti-OVOL1 polyclonal antibody (Abcam, Cambridge, MA, USA), rabbit anti-OVOL2 monoclonal antibody (Abcam, Cambridge, MA, USA) or mouse anti-GAPDH monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) were used for BRCA1, BRCA2 and β-actin level detection separately. The specific protein-antibody complex was detected by incubating with horseradish peroxidase conjugated goat anti-rabbit (Abcam, Cambridge, MA, USA) or rabbit anti-mouse IgG (Abcam, Cambridge, MA, USA). The chemiluminescence reaction was carried out using the ECL kit (Pierce, Appleton, WI, USA).

Dual luciferase assay

A segment of 332 bp DNA of the ZEB1 promoter region was cloned into upstream of firefly luciferase coding region in pGL3-basic vector (Promega, Madison, WI, USA) to generate luciferase reporter vector.

HEK293T cells were seeded in 48-well plates and allowed to attach overnight. OVOL1 expression vector or siRNA was transfected with 100 ng of reporter vector by Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). Empty plasmid or sequence-scrambled short RNA was used as control. Cells were harvested, lysed, and assayed using Dual-Luciferase Assay kit (Promega, Madison, WI, USA) two days after transfection. Each treatment was performed in triplicate in three independent experiments. The results were expressed as relative luciferase activity (Firefly LUC/Renilla LUC).

Cell proliferation assay

SCC-152 cells were seeded in 96-well plates at a density of 5×10^3 cells per well in RPMI-1640 medium, and allowed to attach overnight. OVOL1 expression vector or siRNA was

transfected into cells, with empty plasmid or sequence scrambled short RNA as control. Twenty microliters MTT (5 mg/ml) (Sigma, St. Louis, MO, USA) were added into each well two days after transfection, and incubated for further 2 hours. The absorbance was recorded at A570 nm with a 96-well plate reader after DMSO addition.

Cell apoptosis assay

Cell apoptosis was measured using Annexin V-fluorescein isothiocyanate/prepidium iodide (PI) double staining system (BD Bioscience) according to the manufacturer's procedure.

Cell migration and invasion assay

3×10^4 SCC-152 cells were seeded into 12-well plates and allowed to attach overnight. The cells were transfected with siRNA, OVOL1, or ZEB1 overexpression vector for 48 hours. Cells were harvested and seeded in the top chamber of a typical 24-well transwell plate, and incubated for 12 hours. Filters were then submerged in formalin for 15 min and cells stained by hematoxylin-eosin and calculated. For cell invasion assays, the procedure was similar to the cell migration assay, except transwell membranes were pre-coated with 24 µg/µl Matrigel (BD Bioscience, Franklin Lakes, NJ, USA).

Chromatin Immunoprecipitation (ChIP)

5×10^6 cells were used for each assay. Cells were first cross-linked by 1% formaldehyde at room temperature for 5 minutes and then resuspended in lysis buffer [1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.1)] and incubated for 15 minutes on ice. After being sonicated and centrifuged at 13,000 rpm at 4°C for 10 minutes, the supernatant of the lysate was diluted 10 times in the dilution buffer [0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, 16.7 mM Tris-HCl (pH 8.1)] and was incubated for 1 hour on a rotating platform at 4°C with Protein-A Sepharose. The precleared chromatin was incubated with 1 mg OVOL1 atibody or IgG for 8 hours at 4°C and the immune complexes were collected with Protein-A Sepharose. After three washes with the LiCl Buffer [0.25 M LiCl, 1% Triton X-100, 1% deoxycholic acid, 1 mM EDTA, 10 mM Tris-HCl (pH 8.1)] and twice with the TE buffer [1 mM EDTA, 10 mM Tris-HCl (pH 8)], DNA was extracted by phenol-chloro-
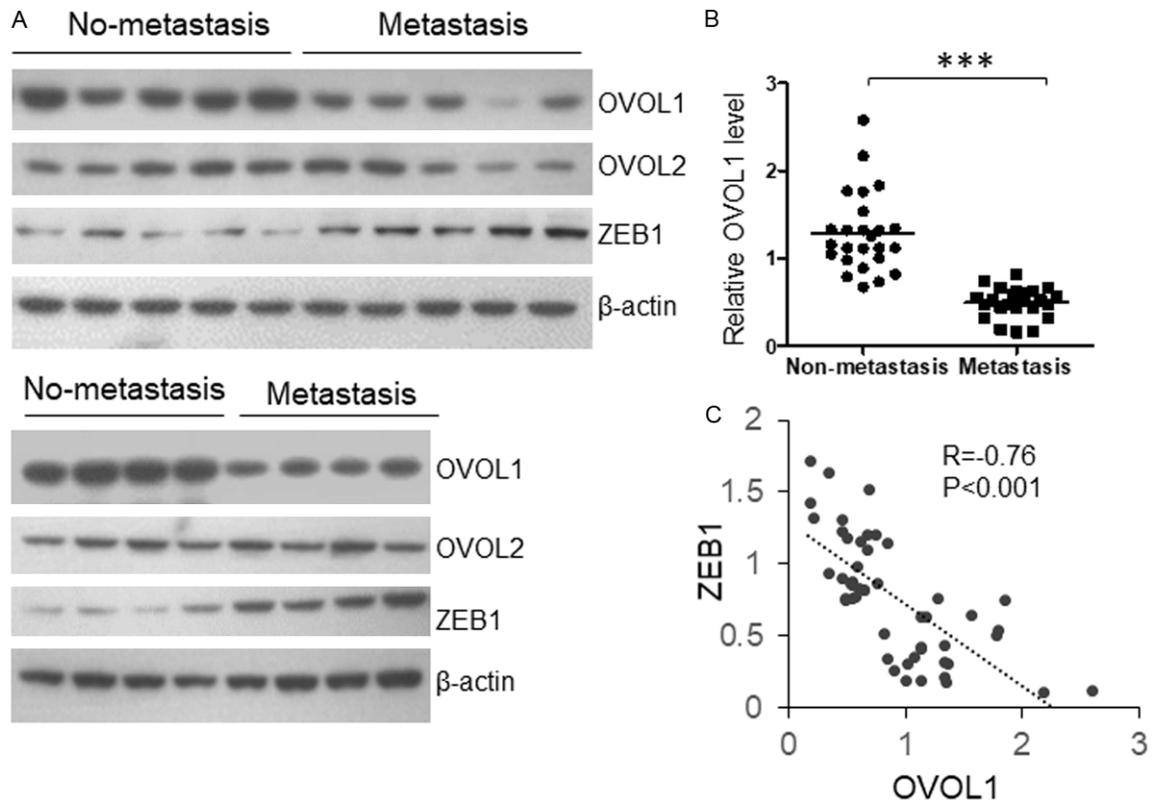
Figure 1. OVOL1 level is reduced in metastatic OSCC samples and negatively correlated with ZEB1 protein level. OSCC tissue samples were collected from 25 metastatic and 25 non-metastatic OSCC patients. The protein levels of OVOL1 and ZEB1 were determined by immunoblotting, and results were quantified by using Quantity One.

form and precipitated by ethanol. Quantitative real-time PCR was performed with SYBR-Green PCR mix.

Statistical analysis

Results were analyzed using SPSS Statistical Package version 15. Student's t-test was used to analyze independent two groups and χ^2-analysis was used to analyze the correlation. P<0.05 was considered significant.

Results

To investigate the roles of OVOL proteins during the progression of OSCC, we collected clinical samples from 50 OSCC patients (25 non-metastasis and 25 lymph node metastasis). Protein was extracted and the levels of OVOL1, OVOL2 and ZEB1 were examined by western blot in 18 OSCC patients (9 non-metastasis and 9 lymph node metastasis) and then the level of OVOL1 and ZEB1 was detected in a further group of 32 OSCC patients (16 non-metastasis and 16 lymph node metastasis). The relative protein levels were quantified and results

were analyzed using Student's t-test. As shown in **Figures 1A**, **1B**, and S1, the OVOL1 level was significantly reduced in the metastatic OSCC samples with a statistical power higher than 0.9. Because of a reported negative regulatory relationship between OVOL1 and ZEB1 in other cancer cells, we wanted to know whether the ZEB1 expression was regulated by OVOL1 in OSCC patients. The ZEB1 protein level was detected and we observed increased ZEB1 level in metastatic OSCC tissues (**Figure 1A**) and a significant negative correlation existed between OVOL1 and ZEB1 protein level (**Figure 1C**).

It is reported that OVOL1 binds a site in the promoter region of ZEB1, +320 to 340 [4]. We did the Chip assay by using OVOL1 antibody and SCC-152 cells to investigate this relationship in OSCC. As shown in **Figure 2A**, OVOL1 was successfully recruited by OVOL1 antibody. PCR product b (+382 to +530) was 23.13 fold higher than the IgG control, but PCR product a (-765 to 658) was not recruited by OVOL1 antibody, indicating that the binding site is located near the product b region, probably +320 to 340.
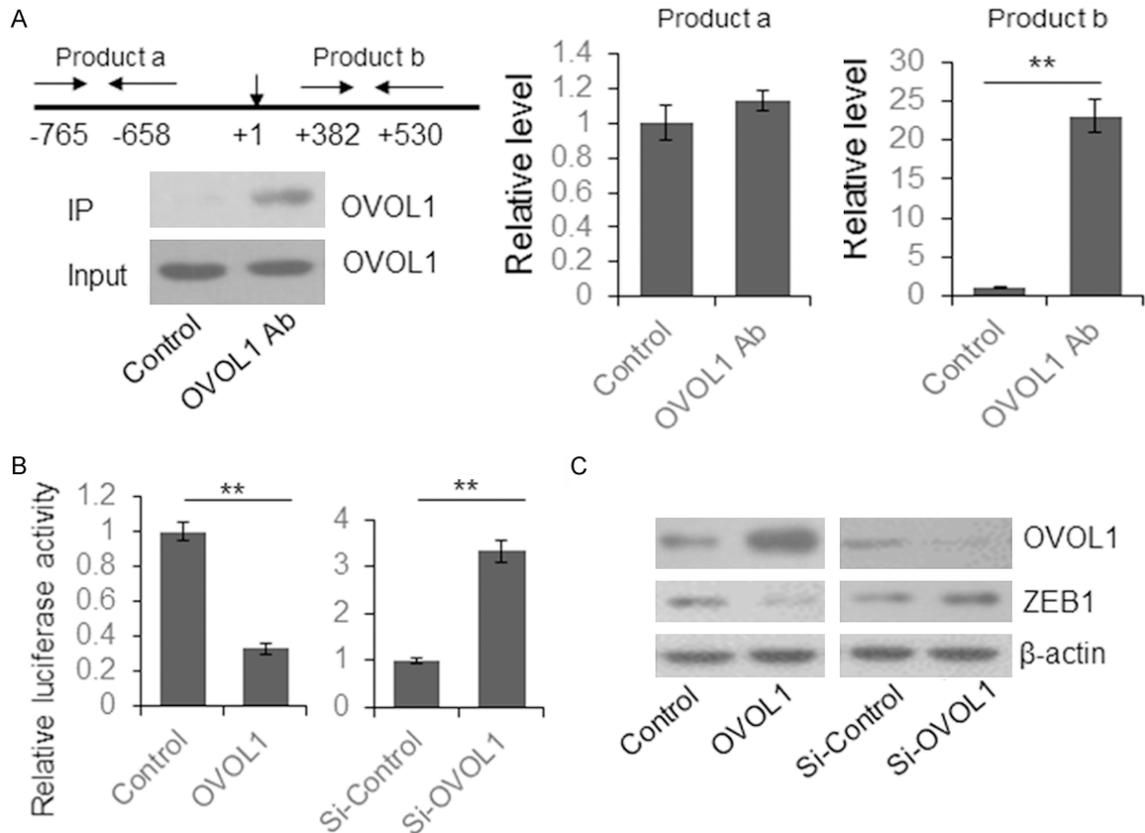
Figure 2. OVOL1 repressed ZEB1 expression by direct binding to the promoter region. A. The binding of OVOL1 to ZEB1 promoter region was detected by ChIP assay. The protein level of OVOL1 in the input and IP samples were determined by immunoblotting. Two segments near the ZEB1 promoter region were quantified by qRT-PCR. B. The effect of OVOL1 binding to the ZEB1 promoter region was determined by dual luciferase assay. C. The OVOL1 was overexpressed or knocked down in SCC-152 cells and the ZEB1 level was determined by immunoblotting.

To further identify the regulatory modules of OVOL1 and ZEB1, a 332 bp region of ZEB1 promoter region containing the OVOL1 binding site was cloned into pGL3-basic vector to construct a reporter plasmid. HEK293T cells were transiently transfected with ZEB1 reporter vector with OVOL1 expression or control vector for 48 hours, and then lysed for dual luciferase assay. We observed a significantly reduced luciferase activity in the OVOL1 overexpression group. When the endogenous OVOL1 was knocked down by siRNA, the luciferase activity was increased (**Figure 2B**). To further verify the repressive effect of OVOL1 on ZEB1 at the protein level, OVOL1 was overexpressed or knocked down in SCC-152 cells. The OVOL1 and ZEB1 levels were determined by immunoblotting. We observed reduced ZEB1 level in the OVOL1-overexpressed cells and increased ZEB1 level in the OVOL1-knocked down cells (**Figure 2C**).

To explore the biologic function of OVOL1 in OSCC, OVOL1 was overexpressed or knocked down in SCC-152 cells, and the cell viability was determined by MTT assay. We observed decreased cell viability in the OVOL1 overexpressed cells; and in the OVOL1 knocked down cells, the cell viability was significantly increased (**Figure 3A**). Cell apoptosis was determined by Annexin V antibody-based flow cytometry. The number of apoptotic cells was increased in the OVOL1 overexpression group and reduced in OVOL1 knocked down group (**Figure 3B**). Subsequently, the cell migration and invasion were determined by transwell assay. As shown in **Figure 3C** and **3D**, the OVOL1 overexpressed SCC-152 cells have increased migration and invasive cell number, and OVOL1 knocked down cells have decreased migration and invasive cell number. These results indicated that OVOL1 functions as a tumor suppressor in OSCC.

To further identify whether OVOL1 represses OSCC growth by regulating ZEB1, we constructed a ZEB1 expression vector. SCC-152 cells
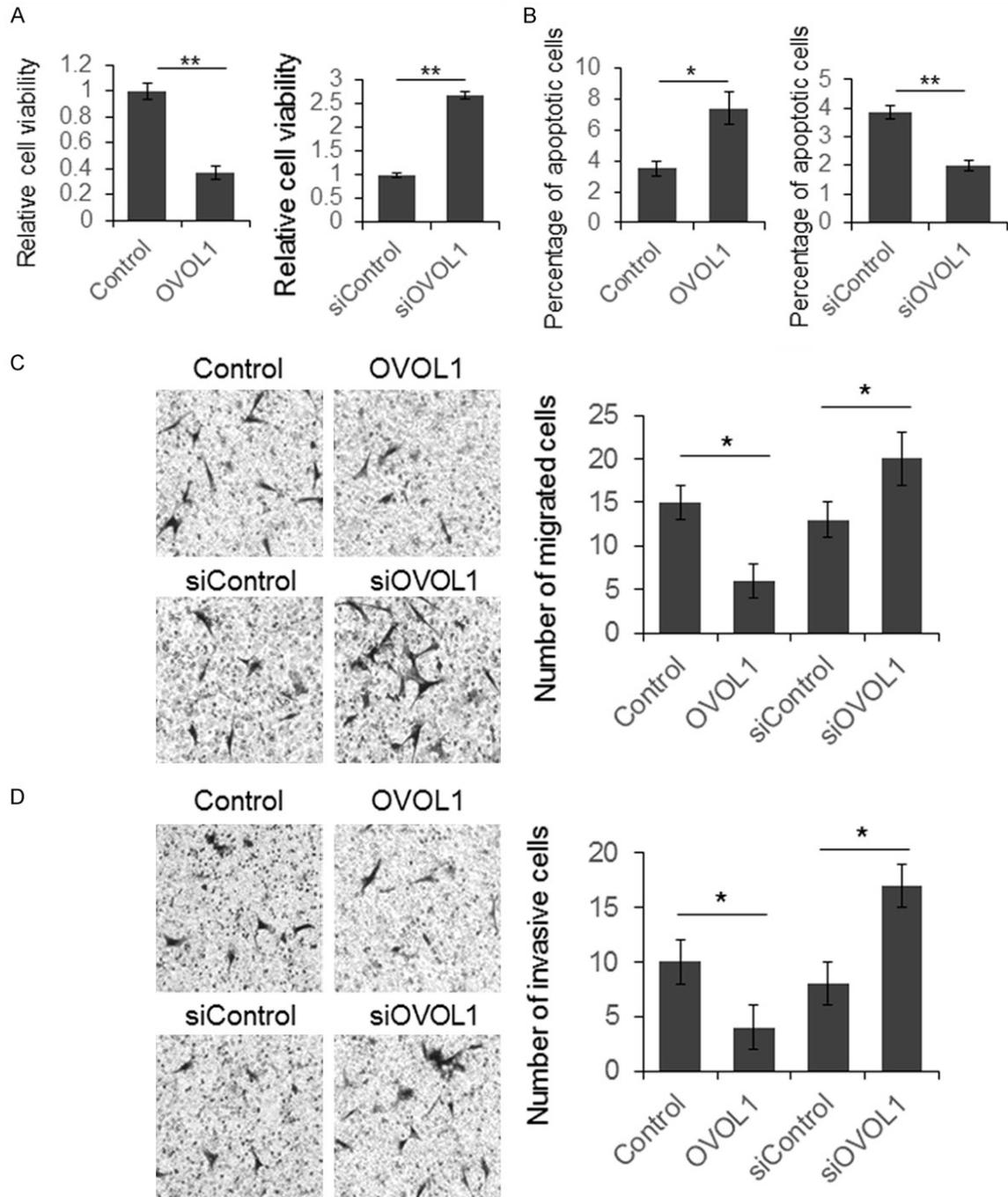
Figure 3. OVOL1 repressed OSCC cell proliferation, migration, invasion, and induced cell apoptosis. SCC-152 cells were transfected with OVOL1 expression vector or siRNA for 48 hours and then the cells were subjected to proliferation (A), apoptosis (B), migration (C) and invasion (D) assays. Results were analyzed by Student t test and P<0.05 was considered significant. *P<0.05, **P<0.01.

were transfected with OVOL1 and ZEB1 expression vector or just OVOL1 expression vector for 48 hours, with cells transfected with empty vector as control. The cell viability, apoptosis, migration, and invasion capacity were detected. As shown in **Figure 4A**, the repressed cell

viability caused by OVOL1 was almost fully rescued by ZEB1. Meanwhile, the up-regulated cell apoptosis by OVOL1 was partially reduced by ZEB1 (**Figure 4B**). The ZEB1 overexpression also rescued the repressive function of OVOL1 on cell migration and invasion (**Figure 4C** and **4D**).
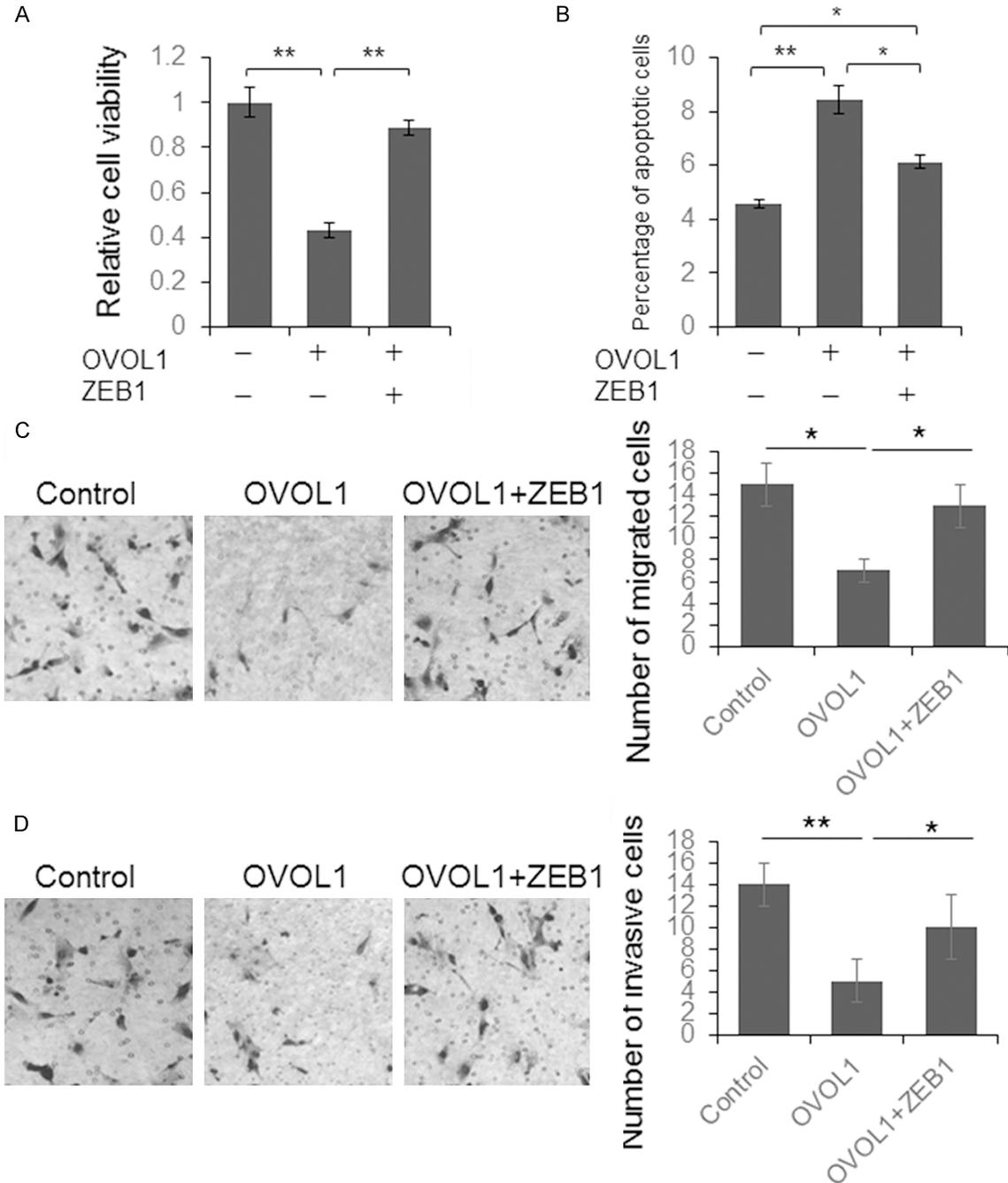
Figure 4. ZEB1 partially rescued the tumor suppressor function. SCC-152 cells were transfected with OVOL1 expression vector, or OVOL1 plus ZEB1 vectors for 48 hours and then the cells were subjected to proliferation (A), apoptosis (B), migration (C) and invasion (D) assay. Results were analyzed by Student t test and P<0.05 was considered significant. *P<0.05, **P<0.01.

Discussion

Lymph node metastasis is the primary cause of death in OSCC patients, so understanding the underlying molecular mechanism is critical. OVOL1 is a transcription factor, that functions as a "break" to repress the cell metastasis in breast and prostate cancers [4]. To explore the role of OVOL1 in the progression of OSCC, especially during the metastasis progress, we first detected the protein level of OVOL1 and OVOL2 in the metastases and non-metasta-

tic OSCC tissues. We found OVOL1 level was increased significantly in the non-metastatic OSCC tissues and negatively correlated with ZEB1 level. Through ChIP and dual luciferase assay, we confirmed that OVOL1 repressed ZEB1 expression by directly binding to the promoter region of ZEB1. Subsequent functional study confirmed that OVOL1 functioned as a tumor suppressor, suppressed SCC-152 cell proliferation, migration, and invasion and promoted cell apoptosis. Interestingly, ZEB1 almost fully rescued the overexpressed OVOL1 function in SCC-152 cells.

ZEB1 is a transcription factor which can inhibit E-cadherin expression. Previous studies indicated that ZEB1 overexpression was significantly correlated with aggressiveness and poor clinical prognosis in many kinds of human malignancies including OSCC [7-9]. Our present study confirmed the negative correlation between OVOL1 and ZEB1 in OSCC clinical samples and cells for the first time. Furthermore, we confirmed that OVOL1 repressed OSCC cell proliferation, migration, and invasion mainly through inhibiting ZEB1 expression.

In summary, our study found that higher OVOL1 expression contributes to the progression of OSCC through inhibiting ZEB1, which may provide markers for prognostic prediction in OSCC.

Acknowledgements

Disclosure of conflict of interest

None.

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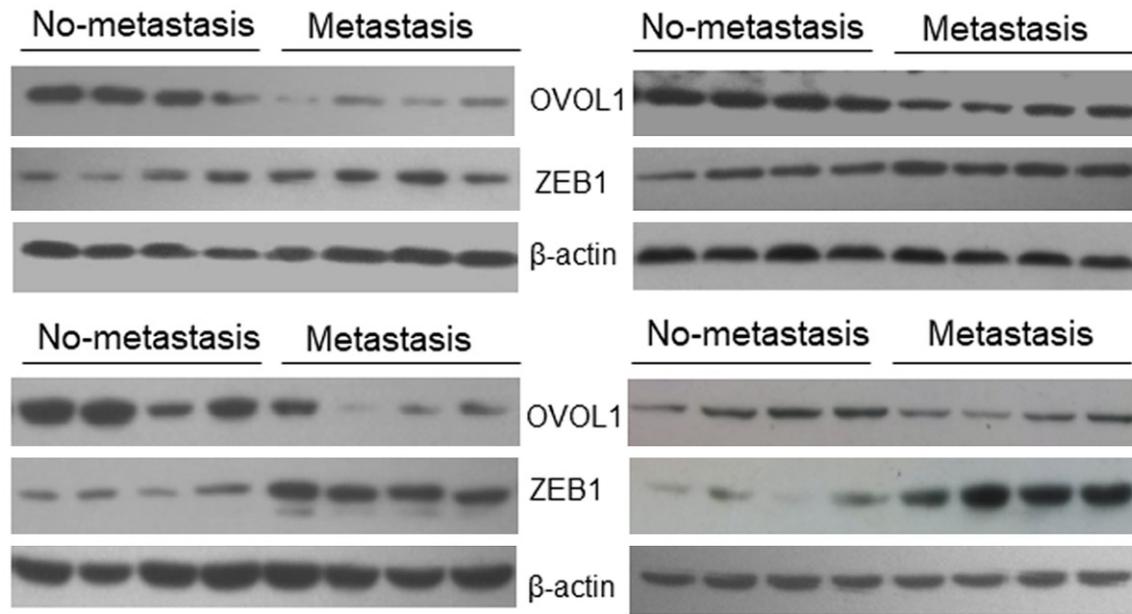
Figure S1. The level of OVOL1 and ZEB1 of 32 collected clinical samples from 32 OSCC patients (16 non-metastasis and 16 lymph node metastasis). The level of OVOL1 is reduced significantly with P<0.001 and power >0.9.