

Knockdown of ectodysplasin-A receptor-associated adaptor protein exerts a tumor-suppressive effect in tongue squamous cell carcinoma cells

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Received May 12, 2019; Accepted February 10, 2020

DOI: 10.3892/etm.2020.8578

Abstract. Tongue squamous cell carcinoma (TSCC) is a common malignancy in oral cancer with a high mortality and morbidity. The ectodysplasin-A receptor-associated adaptor protein (EDARADD) is a death domain-containing adaptor protein that interacts with the TNF family ligand ectodysplasin A receptor. It is known that EDARADD has an effect on the development of ectodermal derivative tissues, such as hair and teeth. EDARADD expression is also associated with the development of melanoma. However, the role of EDARADD in TSCC remains unknown. The aim of the present investigation was to explore whether EDARADD plays a role in the biological function of TSCC. Immunohistochemistry was used to measure the expression of EDARADD in TSCC tissues and adjacent normal tissue. *EDARADD* was knocked down in a TSCC cell line *in vitro* using a specific lentivirus. The expression level of the EDARADD gene and the efficacy of gene knockdown were evaluated by reverse transcription-quantitative PCR, while EDARADD protein expression and the expression levels of Bcl-2, MYC and NF- κ Bp65 were determined by western blotting. Additionally, MTT assays, colony formation assays and apoptosis assays were carried out to examine the effect of EDARADD knockdown on the TSCC cells. A previous study showed that the majority of the TSCC tissues that were tested had high EDARADD expression. The expression of EDARADD both at mRNA and protein levels was significantly lower ($P<0.01$) after the gene was knocked down in the CAL27 cells compared with the level in control cells. Downregulation

of EDARADD expression inhibited colony formation and proliferation and induced apoptosis of CAL27 cells when compared to control cells ($P<0.01$). Taken together, these results suggested that EDARADD may be actively involved in the progression of TSCC and that EDARADD may be a novel therapeutic target for the treatment of TSCC.

Introduction

Oral cancer is one of the most common solid cancers and an estimated 300,000 new cases were diagnosed and 145,000 related deaths were caused worldwide in 2012 (1). Among the different types of oral cancer, one of the most common malignancies that poses a threat to human health and life is tongue squamous cell carcinoma (TSCC), accounting for approximately 25-40% of cases of oral cancer (2-4). TSCC is characterized by high malignancy, invasive growth and early lymph node metastasis (5). Current therapeutic strategies for TSCC include traditional surgical resection, radiotherapy and chemotherapy. Despite advances in these treatments, there has been no significant improvement in the prognosis and survival rate of patients with TSCC over the past 10 years, due to lymphatic metastasis, mortality and recurrence (6,7). The rates of incidence and death remain high (8,9) and the overall 5-year survival rate is less than 60% (10,11). In addition, traditional treatments may harm the functioning of surrounding organs leading to a decline in patient quality of life, due to potential difficulties in chewing, swallowing and speech (5). In-depth research is urgently required to determine the precise molecular mechanism underlying TSCC tumorigenesis, which may help to more accurately locate and effectively inhibit cancer cell proliferation during treatment, potentially an effective and practical strategy to improve clinical outcomes. Targeted therapeutic approaches are being developed to improve clinical outcomes based on the identification of potential new biomarkers and therapeutic targets for cancer (12-15).

The ectodysplasin-A receptor-associated adaptor protein (*EDARADD*) gene encodes a protein containing a death domain that interacts with the ectodysplasin-A receptor (EDAR), which is part of the tumor necrosis factor (TNF) receptor family. EDARADD was originally reported to be primarily associated with anhidrotic ectodermal dysplasia, an ectodermal

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Key words: ectodysplasin-A receptor-associated adaptor protein, tongue squamous cell carcinoma, NF- κ Bp65, MYC, Bcl-2

differentiation disorder involving aberrant development of the exocrine sweat glands, teeth and hair (16,17). Related studies have found that EDARADD is not only associated with the occurrence of dental caries, but also affects the development of tooth morphology and tooth quantity (18,19). In the present study, EDARADD was identified to be clinically relevant to head and neck squamous cell carcinoma (HNSCC) through The Cancer Genome Atlas (TCGA) data portal analysis. To assess the clinical significance of EDARADD in TSCC and its potential molecular mechanisms, EDARADD was knocked down in *in vitro* studies using interference technology to identify whether EDARADD affects the tumorigenicity of TSCC cells.

Materials and methods

Gene information. UALCAN database is a publicly available interactive online portal used to perform in-depth analyses of TCGA gene expression data (20), (<http://ualcan.path.uab.edu/>), TCGA analysis was used to search for the expression of the EDARADD gene in the HNSCC and generate a box-line diagram of EDARADD expression levels in 40 normal individuals and 520 patients with HNSCC.

Patients and tissue specimens. Specimens and histologically normal peri-tumor tissues that had been collected from patients operated on for primary TSCC between January 2016 and December 2018 at the Department of Oral Medicine, Central Hospital of Xuzhou, The Xuzhou Clinical College of Xuzhou Medical University (Xuzhou, China) were analyzed in the current study after obtaining informed written consent from each patient. The tissue samples were immediately frozen in liquid nitrogen for further use. The present study was approved by the Ethics Committee of the Central Hospital of Xuzhou, The Xuzhou Clinical College of Xuzhou Medical University (reference no. 2009XL002) and was conducted according to The Declaration of Helsinki. A total of 33 patients were enrolled in the present study (age, 28-87 years; mean age, 56.9 years), 22 (66.7%) were men and 11 (33.3%) were women. The tumor samples were evaluated according to the World Health Organization stage and grading system (21).

Immunohistochemistry (IHC). Paraffin-embedded sections of the tissue samples (4- μ m-thick) were deparaffinized and rehydrated before antigen retrieval was performed in citrate buffer (pH 6.0; cat. no. G1202; Wuhan Servicebio Technology Co., Ltd) for 10 min at 100°C. Thereafter, the preparations were incubated with 3% H₂O₂ and 3% BSA (cat. no. G5001; Wuhan Servicebio Technology Co., Ltd.) for 20 min at 25°C, in order to block endogenous peroxidase activity. The sections were then incubated with primary rabbit anti-human monoclonal EDARADD antibody overnight at 4°C (1:200; cat. no. D123818; Sangon Biotech (Shanghai) Co., Ltd.), followed by incubation with a horseradish peroxidase-labeled goat anti-rabbit secondary antibody for 30 min at 37°C (1:200; cat. no. GB23204; Wuhan Servicebio Technology Co., Ltd.). The slides were then incubated with diaminobenzidine (cat. no. G1211; Wuhan Servicebio Technology Co., Ltd.) for 20 min at 25°C and finally counterstained with hematoxylin for 3 min at 25°C (cat. no. G1004; Wuhan Servicebio

Technology Co., Ltd.). As a negative control, PBS was used instead of the primary antibody for one slide. All the stained slides were examined by two independent pathologists, who were blinded to patient clinical information. A total of three slices of each sample of TSCC tissues and adjacent tissues were selected and five fields were observed under the microscope (Olympus BX53; Olympus Corporation). The positive staining area was processed quantitatively using Image-Pro Plus Version 6.0 software (Media Cybernetics, Inc.). The immunostaining intensity was classified into four categories: A score of 3 indicated strong staining, 2 indicated moderate staining, 1 indicated weak staining and 0 indicated no staining. Finally, the data were divided into two categories: Low expression (weak or no immunoreactivity) and high expression (strong or moderate immunoreactivity).

Cell line and culture conditions. 293T cells and the human TSCC cell lines CAL27, SCC25 and SCC9 were used in this study. The cell lines were purchased from the The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. The CAL27, SCC25, SCC9 and 293T cells were grown in DMEM (Corning, Inc.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.). 293T cells were grown in DMEM without FBS 2 h prior to transduction. The cells were maintained at 37°C in a humidified incubator at 5% CO₂ and 95% air (Sanyo; Panasonic Corporation).

Short hairpin (sh)RNA lentivirus infection. shRNA for the inhibition of EDARADD expression was purchased from Shanghai GeneChem Co., Ltd. The interference target sequence of the EDARADD shRNA was 5'-GTACTTGTTCTCCTGCTT-3' (shEDARADD) and the sequence used for the negative control (shCtrl) was 5'-TTCTCCGAACGTGTCACGT-3'. Lentiviral titers were determined by reverse transcription-quantitative PCR (RT-qPCR; as described below) and transfected into CAL27 cells using a lentiviral vector (GV 112; Shanghai GeneChem Co., Ltd) with a multiplicity of infection of 20. The cells were inoculated in six-well plates with 2 ml cell suspension at a density of 2x10⁵ cells/ml and incubated overnight; the spent medium was replaced with fresh medium to continue the culture. After 72 h of incubation, the cells were observed under a fluorescence microscope (Olympus Corporation) and the successfully-infected cells were positive for green fluorescent protein. The interference efficiency of the EDARADD shRNA was measured by RT-qPCR and western blotting (as described below).

Western blotting. Total protein was collected from the TSCC cell lines using cell lysis buffer (P0013B; Beyotime Institute of Biotechnology), and the protein concentration was determined using a BCA protein detection kit (P0010S; Beyotime Institute of Biotechnology). A total of 30 μ g of each protein sample was separated on an SDS-PAGE (10% gel) and transferred onto a PVDF membrane at an electric current of 300 mA for 2.5 h. The membrane was blocked for 1 h at 25°C using 5% non-fat dry milk in TBST with 0.05% tween20 and incubated overnight at 4°C with diluted primary rabbit anti-human monoclonal antibodies against EDARADD (cat. no. orb183301; 1:100; Biorbyt Ltd.), MYC (cat. no. ab32072; 1:1,000; Abcam) and

NF- κ Bp65 (cat. no. 8242; 1:500; Cell Signaling Technology, Inc.) and primary mouse anti-human monoclonal antibodies against Bcl-2 (cat. no. ab692; 1:500; Abcam) and GAPDH (cat. no. sc-32233; 1:2,000; Santa Cruz Biotechnology, Inc.). After washing three times with PBS, the membrane was incubated with secondary anti-mouse IgG antibody (cat. no. 7076; 1:2,000; Cell Signaling Technology, Inc.) or anti-rabbit IgG antibody (cat. no. 7074; 1:2,000; Cell Signaling Technology, Inc.) at room temperature for 1.5 h. The protein levels were visualized with Pierce™ ECL reagent (Thermo Fisher Scientific, Inc.) and developed onto X-ray film.

RNA extraction and RT-qPCR. Total RNA was isolated from the TSCC cell lines and purified using SuPerfecTRI™ reagent (Shanghai Pufei Biotechnology Co., Ltd.) according to the manufacturer's instructions. The mRNA fragments were used to generate cDNAs using M-MLV reverse transcriptase (Promega Corp.) for 60 min at 37°C according to the manufacturer's protocol. The abundance of the EDARADD mRNA in the CAL27, SCC25 and SCC9 cells was then detected by qPCR using the Maxima SYBR Green qPCR Master Mix (Takara Bio, Inc.) by a two-step method. The cycling conditions were as follows: 95°C for 10 min, then 40 cycles at 95°C for 15 sec and 60°C for 60 sec. The reverse transcription primer and microRNA PCR primers were purchased from Guangzhou Ruibo Biotechnology Co., Ltd. The relative change in the mRNA expression levels was calculated by the comparative threshold cycle method ($2^{-\Delta\Delta C_q}$) (22) using the GAPDH as an internal reference gene control. The experiment was repeated three times. The sequences of the primers used are as follows: EDARADD forward, 5'-GACCAACCCAAAGAGGACAG-3' and reverse, 5'-CCAGAATGATGAGGCACCAT-3'; GAPDH forward, 5'-TGACTTCAACAGCGACACCA-3' and reverse, 5'-CACCCTGTTGCTGTAGCCAAA-3'.

Cell counting assay. The TSCC cells (2×10^3 cells/well) were plated in 96-well plates and cultured in 5% CO₂ at 37°C for 24 h. After the incubation period, a Celigo cytometer (Beckman Coulter, Inc.) was used to read the plates once a day for 5 consecutive days. The number of cells with cell viability was accurately calculated and statistically analyzed based on the quantity of green fluorescence protein. Using the cell count-fold value, which indicated the cell count at each time point relative to the mean of day 1, cell growth curves were drawn to represent changes in cell proliferation.

MTT assay. The lentivirus-transduced TSCC cell lines were incubated in 96-well plates at a density of 1,500 cells/well for 24 h. The cells were then cultured with 20 μ l (5 mg/ml) MTT (Gen-view Scientific, Inc.) at 37°C and 5% CO₂ for 4 h. After the incubation period, the medium was removed and replaced with 100 μ l of dimethyl sulfoxide for 15 min at room temperature. The absorbance was measured on a microplate reader (Tecan Group Ltd.) at 490 nm. The assay was performed in triplicate.

Annexin V-allophycocyanin (APC) apoptosis assay. A total of 2×10^5 cells/ml were cultured in a 6-well plate after 3 days of transduction. The cell fusion degree was detected to be ~85% on the 5th day following transduction. The cells were

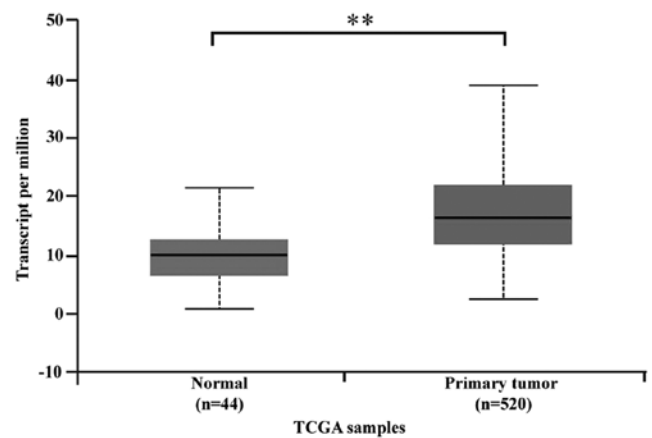


Figure 1. Expression of EDARADD in HNSCC and normal tissues. **P<0.01. EDARADD, ectodysplasin-A receptor-associated adaptor protein; HNSCC, head and neck squamous cell carcinoma.

trypsinized and centrifuged at 4°C for 5 min at 300 x g, washed twice with D-Hanks solution (pH=7.2-7.4) and once with the 1X Binding Buffer, then resuspended in 1X Binding Buffer at a density of 1×10^6 cell/ml. Next, the 100 μ l of the cell suspension were incubated at 25°C for 15 min in the dark with the 5 μ l of eBioscience™ Annexin V Apoptosis Detection Kit APC (Thermo Fisher Scientific, Inc.) Finally, the samples were analyzed in a flow cytometer using Guava easyCyte HT Version 8 system (EMD Millipore).

Colony formation assay. After 3 days of transfection, the cells were disassociated and seeded in six-well plates at a density of 1.5×10^3 cells/well and cultured for ~10 days (medium exchanged every 3 days). The cell clones were observed under a fluorescence microscope. Next, the cells were washed once with PBS before the end of the culture. They were then fixed for 30 min at 4°C by adding 1 ml of 4% paraformaldehyde to each well, followed by washing with PBS and staining with 1,000 μ l Giemsa (Sigma-Aldrich; Merck KGaA) for 15 min at 25°C. The cells were then washed with distilled deionized water several times and a digital camera was used to capture images of the plates. The colonies were observed with an inverted light microscope (Olympus BX53; Olympus Corporation) and quantified using Image-Pro Plus Version 6.0 software (Media Cybernetics, Inc.). The assay was performed in triplicate.

Statistical analysis. The results in this study are presented as the mean \pm standard error of the mean from at least three experiments. Student's t-test was used to analyze the data in two groups with SPSS 21.0 software (IBM Corp). The clinical characteristics data were analyzed by Fisher's exact test. P<0.05 was considered to represent statistical significance.

Results

EDARADD is overexpressed in HNSCC tissues. In the present study, EDARADD expression data were obtained from the online UALCAN database. A box plot of EDARADD expression showed that EDARADD was highly expressed in HNSCC tissues compared to the levels of expression in non-tumor tissues (P<0.01; Fig. 1).

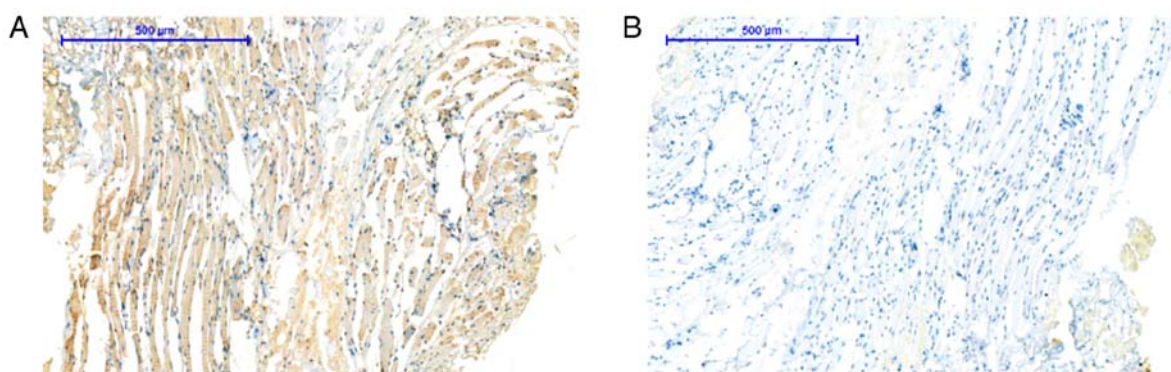


Figure 2. Representative figures of immunohistochemical staining for EDARADD in TSCC tissues and paired adjacent normal tissue. Immunohistochemical staining of (A) TSCC tissues and (B) paired adjacent normal tissue. Magnification, x50. EDARADD, ectodysplasin-A receptor-associated adaptor protein; TSCC, tongue squamous cell carcinoma.

EDARADD expression in TSCC. In the present study, the expression level of EDARADD in TSCC tissues was markedly higher than that in normal adjacent tissues (Fig. 2). The results indicated that the expression level of EDARADD in the normal adjacent tissues was 15.15% (5/33), while the expression rate in TSCC was 60.61% (20/33). The difference was statistically significant ($P < 0.001$) and the results are shown in Table I.

Association between the expression of EDARADD in tumor tissues and clinical and pathological parameters of patients. The IHC results from tumor tissues were compared with clinical data (sex and age) and pathological parameters (TNM and clinical stages). The results showed that the expression levels of EDARADD in TSCC were associated with the degree of tumor differentiation ($P = 0.003$) and local recurrence ($P = 0.027$), as shown in Table II. There were no statistically significant differences in gender, age, TNM staging and tumor size between patients with high or low levels of EDARADD expression ($P > 0.05$).

EDARADD gene expression in TSCC cell lines. Using GAPDH as a standardized internal control, the abundance of EDARADD mRNA in the CAL27, SCC25 and SCC9 cells was evaluated by RT-qPCR (Fig. 3). The results, (SCC25 cells, average $\Delta Ct = 8.58$; CAL27 cells, average $\Delta Ct = 9.21$; SCC9 cells, average $\Delta Ct = 10.99$), suggested there was expression of EDARADD in all three cell lines.

EDARADD mRNA and protein expression in TSCC cell lines. The stable knockdown of EDARADD expression in CAL27 cells was established to assess the potential effects of EDARADD. In the present study, CAL27 cells were successfully infected with the lentivirus expressing shEDARADD or shCtrl (Fig. 4A). Fluorescence microscopy (Olympus IX71; Olympus Corporation) demonstrated that the rates of infection and expression of green fluorescent protein were $> 80\%$. RT-qPCR and western blotting were used to assess the interference efficiency of shEDARADD. As indicated in Fig. 4B and C, there was an evident decrease in the expression of EDARADD at both the mRNA ($P < 0.01$) and protein levels in cells transfected with shEDARADD compared with shCtrl. These results indicated that the efficiency of

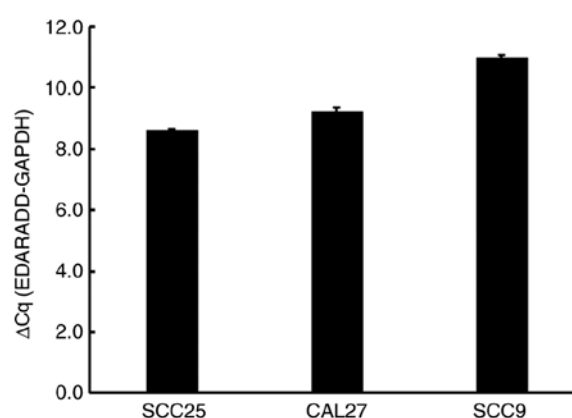


Figure 3. EDARADD gene expression in TSCC cells. EDARADD mRNA levels in different cell types relative to GAPDH. EDARADD, ectodysplasin-A receptor-associated adaptor protein; TSCC, tongue squamous cell carcinoma.

EDARADD knockdown, based on the mRNA expression levels, reached 60.4%, and the endogenous expression of EDARADD gene was reduced at the protein level.

Knockdown of EDARADD affects the cloning ability of TSCC cells. Following the knockdown of EDARADD expression in CAL27 cells, cell counting assays were performed. The results demonstrated that the number of CAL27 cell clones in the CAL27 culture infected with shEDARADD was significantly lower than that in the shCtrl group ($P < 0.01$; Fig. 5), implying that the EDARADD gene contributed to the cloning ability of the CAL27 cells.

Knockdown of EDARADD induces apoptosis in TSCC cell lines. To confirm whether EDARADD expression is associated with the regulation of apoptosis in TSCC cells, flow cytometry analysis was performed. The data indicated that shEDARADD significantly enhanced the apoptotic ability of CAL27 cells when compared with cells of the shCtrl group ($P < 0.01$; Fig. 6), indicating that the EDARADD gene may be involved in the regulation of apoptosis in CAL27 cells.

Knockdown of EDARADD suppresses the proliferation of TSCC cells. To further elucidate the role of EDARADD in

Table I. EDARADD expression in TSCC and adjacent tissues.

Characteristic	N	Expression of EDARADD		P-value
		Low expression, n (%)	High expression, n (%)	
TSCC tissue	33	13 (39.39)	20 (60.61)	<0.001
Adjacent tissue	33	28 (84.85)	5 (15.15)	

Data were analyzed by Fisher's exact test. EDARADD, ectodysplasin-A receptor-associated adaptor protein; TSCC, tongue squamous cell carcinoma.

Table II. Relationship between the expression of EDARADD and clinicopathological features of patients with TSCC.

Characteristic	N (33)	Expression of EDARADD		P-value
		Low expression, n (%)	High expression, n (%)	
Sex				0.714
Male	22	8 (36.36)	14 (63.64)	
Female	11	5 (45.45)	6 (54.55)	
Age (years)				0.485
≤60	13	4 (30.77)	9 (69.23)	
>60	20	9 (45.00)	11 (55.00)	
Tumor size (cm)				0.728
≤5	16	7 (43.75)	9 (56.25)	
>5	17	6 (35.29)	11 (64.71)	
TNM stage				0.263
I+II	24	11 (45.83)	13 (54.17)	
III+IV	9	2 (22.22)	7 (77.78)	
Tumor grade				0.003
G1	14	10 (71.43)	4 (28.57)	
G2/G3	19	3 (15.79)	16 (84.21)	
Lymph node metastasis				0.284
Yes	18	9 (50.00)	9 (50.00)	
No	15	4 (26.67)	11 (73.33)	
Distant metastasis				0.276
Yes	13	7 (53.85)	6 (46.15)	
No	20	6 (30.00)	14 (70.00)	
Local recurrence				0.027
Yes	21	5 (23.81)	16 (76.19)	
No	12	8 (66.67)	4 (33.33)	

Data were analyzed by Fisher's exact test. EDARADD, ectodysplasin-A receptor-associated adaptor protein; TSCC, tongue squamous cell carcinoma.

the proliferation of TSCC cells, Celigo and MTT assays were performed on CAL27 cells in the shEDARADD and shCtrl groups. The cell-counting results showed that the rate of proliferation in the shEDARADD group was markedly reduced compared to that in the shCtrl group, especially on days 4 and 5 after transfection ($P<0.01$; Fig. 7A and B). Similarly, the MTT assay indicated that the OD 490-fold value of the shEDARADD group was markedly lower on days 4 and 5 as compared to that in the shCtrl group ($P<0.01$; Fig. 7C).

These findings suggest that knockdown of EDARADD may prevent the growth of TSCC cells through inhibition of cell proliferation.

Knockdown of EDARADD inhibits the expression of NF- κ Bp65, MYC and Bcl-2. To identify the molecular mechanism of EDARADD in the regulation of proliferation and apoptosis of TSCC cells, western blotting was performed to determine the expression of NF- κ Bp65, MYC and Bcl-2.

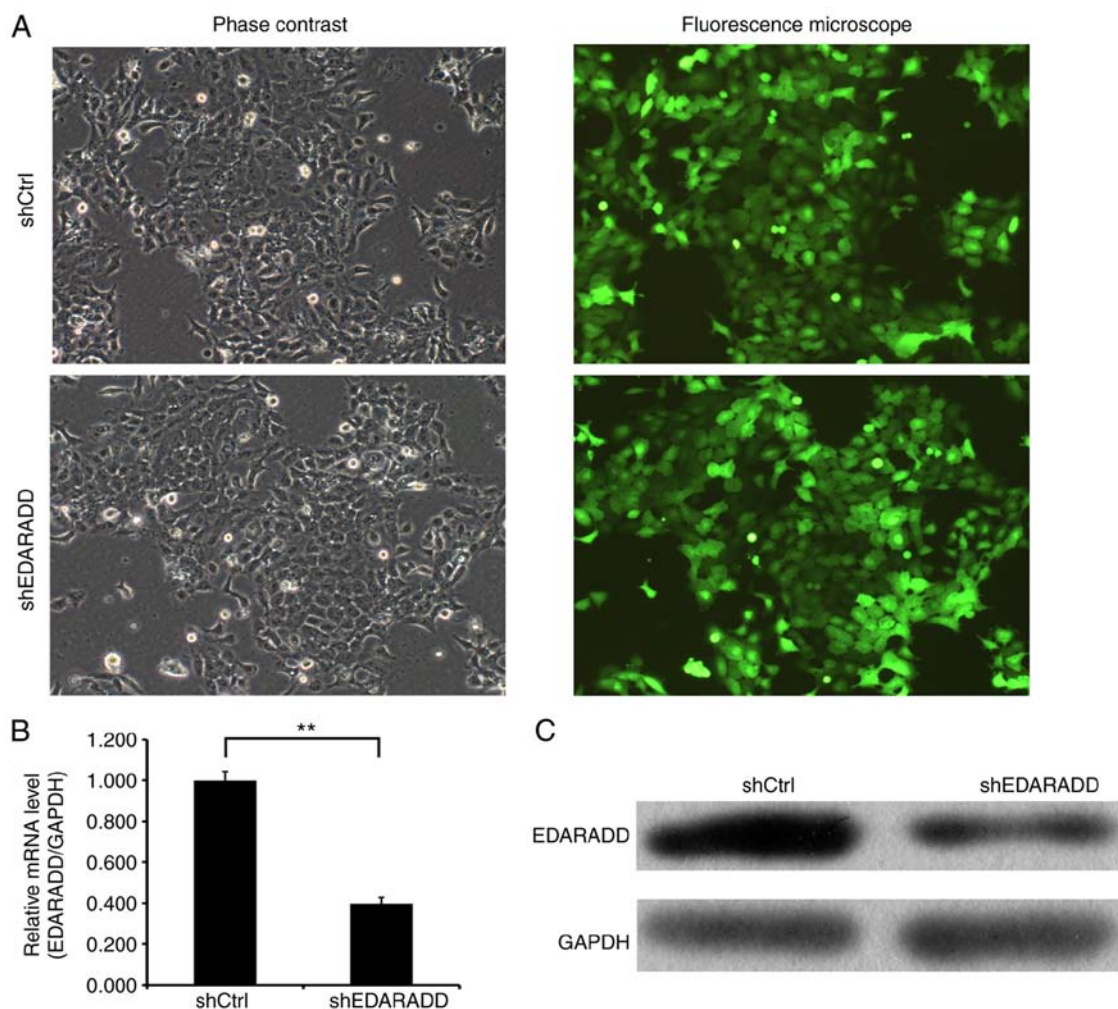


Figure 4. EDARADD mRNA and protein expression in TSCC cells. (A) Fluorescence microscopy. CAL27 cells were successfully infected by lentivirus expressing shEDARADD or shCtrl (magnification, x100). (B) The EDARADD mRNA level in CAL27 cells infected with lentivirus carrying shEDARADD and shCtrl was measured by qPCR. The data are presented as the mean \pm SEM. ** $P < 0.01$. (C) Western blot analysis of EDARADD levels in CAL27 cells transfected with shEDARADD and shCtrl. EDARADD, ectodysplasin-A receptor-associated adaptor protein; sh, short hairpin; TSCC, tongue squamous cell carcinoma; Ctrl, control.

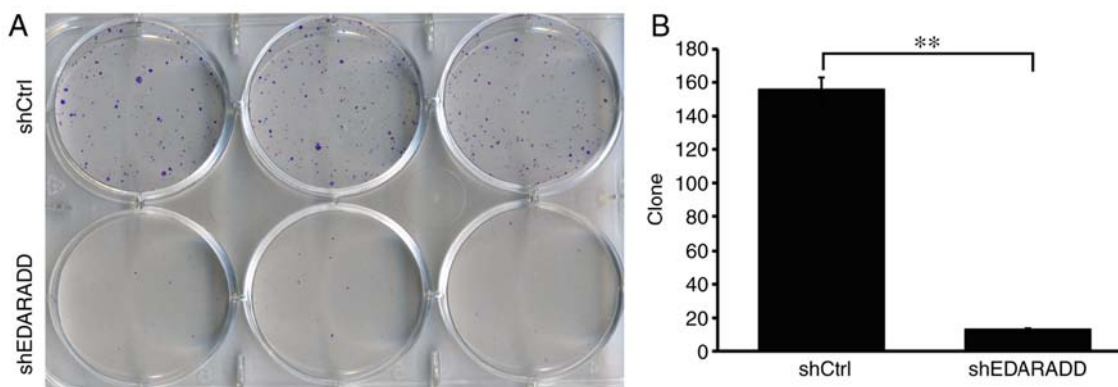


Figure 5. Effects of EDARADD knockdown on the formation of TSCC cell clones. (A) The images of clone-forming assays of the cultures infected with shEDARADD and shCtrl, respectively. (B) The statistical histogram of clone assays. The data are presented as the mean \pm SEM, ** $P < 0.01$. EDARADD, ectodysplasin-A receptor-associated adaptor protein; sh, short hairpin; TSCC, tongue squamous cell carcinoma; Ctrl, control.

As illustrated in Fig. 8, the results suggested that knockdown of the expression of EDARADD in TSCC cells reduced the protein expression of NF- κ Bp65, MYC and Bcl-2 compared with the shCtrl group ($P < 0.05$).

Discussion

TSCC can be caused by a number of factors, such as genetic alterations or environmental factors including tobacco use,

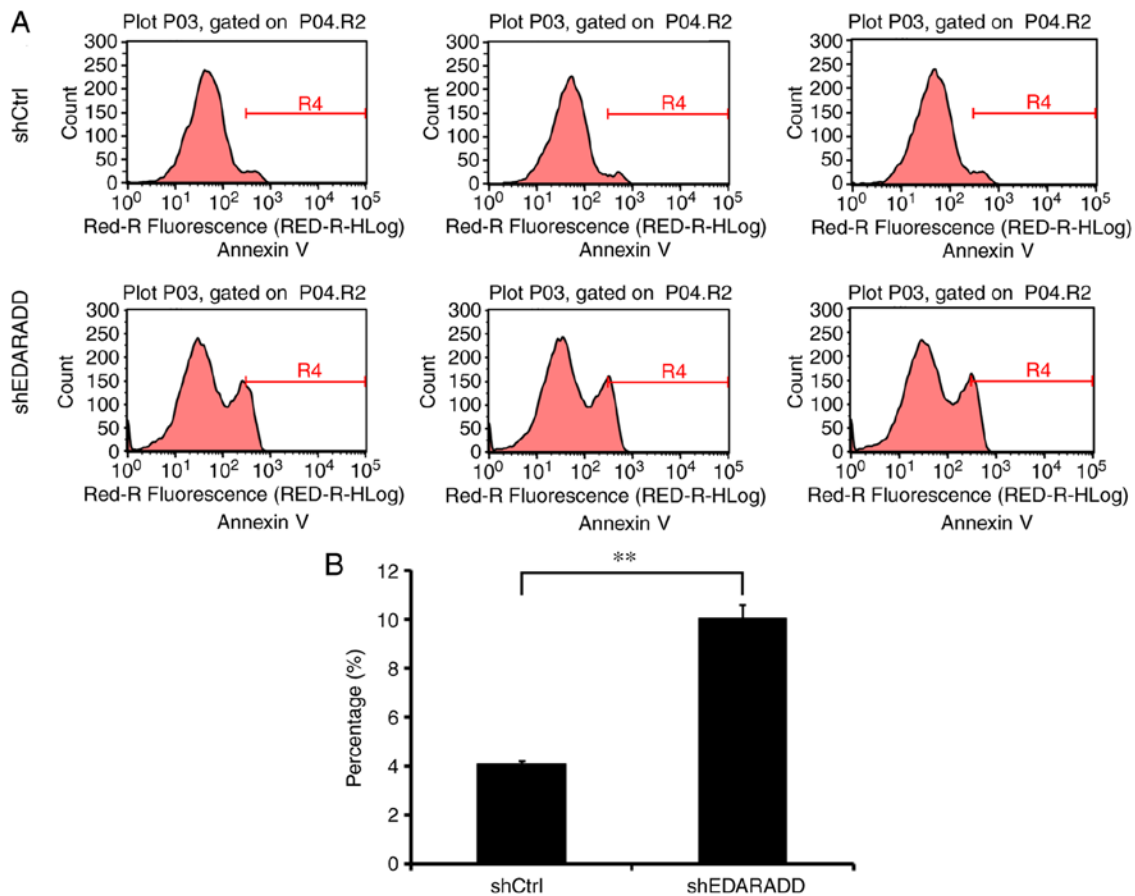


Figure 6. Effects of EDARADD knockdown on the apoptosis of the TSCC cells. Flow cytometry was performed to detect the TSCC cells infected with shEDARADD and shCtrl. (A) The abscissa represents red fluorescence (RED-R-HLog) and the ordinate represents cell count. (B) The data are presented as the mean \pm SEM. Student's t-test was performed for statistical analysis, ** $P < 0.01$. EDARADD, ectodysplasin-A receptor-associated adaptor protein; sh, short hairpin; TSCC, tongue squamous cell carcinoma; Ctrl, control.

alcoholism, chronic inflammation and human papilloma-virus infection (23-25). Recent reports have shown that the incidence of TSCC has increased in young adults (<40 years old), especially in women, with extremely high distant metastasis rate and poorer prognosis (26,27). Therefore, different risk factors leading to TSCC might have distinct molecular mechanism, including the imbalance of cell proliferation and apoptosis (28-32), and mucosal exophytic lesions (33). To the best of our knowledge no previous studies have investigated the biological function of EDARADD in TSCC and its underlying molecular mechanisms.

In the present study, EDARADD expression was identified in HNSCC using TCGA. TSCC is the most lethal and high-incidence head neck squamous cell carcinoma worldwide (34). Therefore, the expression of EDARADD in TSCC was explored further. The expression of EDARADD in TSCC tumor specimens was evidently higher than that in the adjacent normal tissues. In addition, expression of EDARADD was observed in TSCC cell lines. Subsequently, CAL27 cells were selected for shRNA interference studies into the effect of EDARADD on the biological progression of TSCC. Previous studies (35,36) indicated that a lentivirus could be used to effectively infect the CAL27 cells, leading to significant downregulation of gene expression at both the mRNA and protein levels, providing a basis for discovering the novel role of EDARADD in TSCC. Inhibition of EDARADD effectively

enhanced apoptosis and curbed the proliferation of a TSCC cell line, providing evidence for the conclusion that EDARADD has a tumorigenic effect in TSCC.

Few studies have been published that relate to the possible function of EDARADD in tumors. Kumar *et al* (37) reported that the presence of death structures and cell death was observed during EDAR expression and later studies (38) indicated that EDAR was associated with the development of melanoma, and observed that overexpression of EDAR in cells was able to induce cell death. Furthermore, upon ligand binding, EDAR triggers the activation of NF- κ B. EDAR expression leads to apoptosis through an EDARADD and caspase-8 dependent signaling pathway, and hence, it can be defined as a potential tumor suppressor.

EDARADD mutations are associated with the pathogenesis of multiple human disorders (39-43). In mammals, there are two EDARADD isoforms exhibiting different functions encoded by the EDARADD gene, isoforms A and B. Although they exhibit differences in their dynamics, they both activate the NF- κ B pathway (44). Numerous studies (45-47) have shown that NF- κ B transcription factors are adapted to adjust the expression of genes that control cell survival and proliferation. Moreover, deviated activation of the NF- κ B signaling is associated with the propagation of many cancers (48,49). NF- κ B has a dual role in cancer, it not only participates in the immune defense, which can target and eliminate transformed

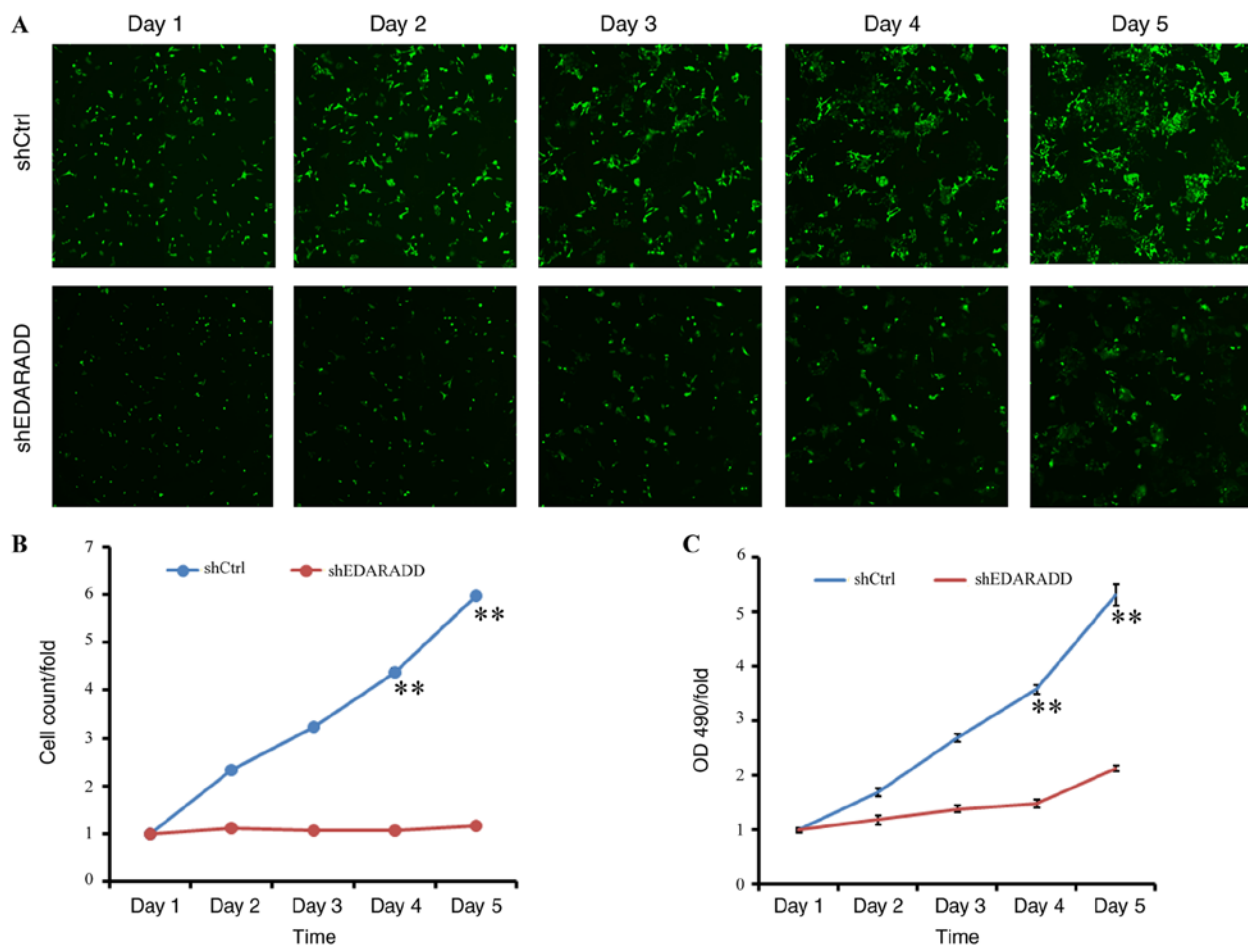


Figure 7. Effects of EDARADD knockdown on TSCC proliferation. (A) CAL27 cells infected with shCtrl and shEDARADD were subjected to the cell counting assay and cell images were taken for 5 consecutive days using the Celigo cytometer. (B) Cell count-fold indicates the cell count of each time point relative to the average cell count of the first day (magnification, x40). (C) CAL27 cells infected with shCtrl and shEDARADD were first subjected to the MTT assay, and then OD values were measured at 490 nm. OD490/fold indicates the OD value of each time point relative to the average value of the first day. The data are presented as the mean \pm SEM. ** $P < 0.01$. EDARADD, ectodysplasin-A receptor-associated adaptor protein; sh, short hairpin; TSCC, tongue squamous cell carcinoma; Ctrl, control.

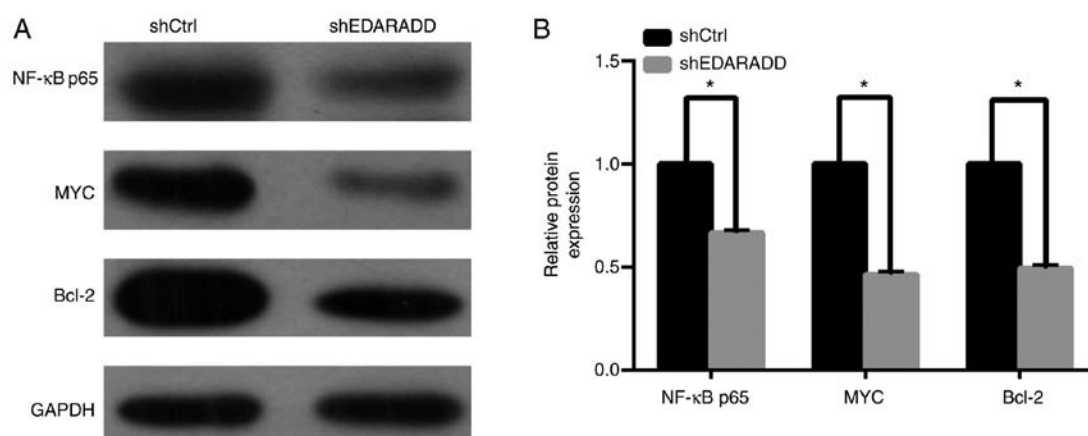


Figure 8. Effects of EDARADD knockdown on the expression of NF- κ Bp65, MYC and Bcl-2. (A) The expression of NF- κ Bp65, MYC, and Bcl-2 was measured by western blotting after EDARADD was knocked down in the TSCC cells. (B) The data are presented as the mean \pm SEM. * $P < 0.05$. EDARADD, ectodysplasin-A receptor-associated adaptor protein; sh, short hairpin; Ctrl, control.

cells, but is also activated in many types of cancer, contributing to the dysregulation of gene expression and prevention of apoptosis and hence, it is responsible for various tumor-promoting functions (50,51).

NF- κ B plays a widespread role in cellular proliferation and acts as a mediator of apoptosis during tumorigenesis, particularly the RelA (p65) subunit (52,53). A recent study proposed that the upregulation of miR-451 selectively downregulated

the expression of NF- κ Bp65 to inhibit the inflammation and proliferation of human glomerular mesangial cells (54). Furthermore, a study reported that penta-acetyl geniposide reduced the protein level of NF- κ Bp65, which has been shown to play a potent pro-apoptotic role in fibroblast-like synoviocytes in adjuvant-induced arthritis in *in vitro* studies (55).

MYC, located downstream of NF- κ B, can stimulate the growth and proliferation of cells, while Bcl-2 family proteins are well known as regulators of apoptosis (56,57). MYC is related to cell growth arrest and proliferation, thereby contributing to tumorigenesis (58). A previous study showed that knocking down MYC resulted in a reduction in the viability of colon cancer cells (59). In addition, a study indicated that the expression of MYC target genes was inhibited in response to the downregulation of Gcn5 histone acetyltransferase activity, contributing to apoptosis of lymphoma cells (60). c-MYC was also found to be repressed in response to long stress-induced non-coding transcript 5 knockdown, which inhibited tumor cell proliferation, thereby stimulating apoptosis of cells (61).

Most anticancer agents assist apoptosis by downregulating the expression of Bcl-2 proteins. For example, triptolide promotes the sensitivity of pancreatic cancer cell line, PANC-1, to gemcitabine by reducing the rates of proliferation and promoting apoptosis, following attenuation of NF- κ B, phosphorylated-p65 and Bcl-2 levels (62). The downregulation of DEAD-box helicase 5 inhibits the expression of Bcl-2 of the NF- κ Bp65-inducible anti-apoptotic factors through TNF- α stimulation (63). Additionally, Wang *et al* (64) demonstrated that the tanshinone analog TC7 markedly induced prostate cancer cell apoptosis by reducing the expression of Bcl-2.

As with most cancer cells, TSCC cells are characterized by imbalanced cell proliferation and apoptosis (65). It has also been reported that the pro-inflammatory transcription factor NF- κ B has a significant effect on the initiation and progression of TSCC. Zhao *et al* (66) reported that c-MYC is likely to drive TSCC progression while Huang *et al* (67) demonstrated that Iroquois homeobox gene 5 can promote the aggressiveness of TSCC cells by activating the NF- κ B signaling pathway, leading to an increase in the nuclear p65 levels and I κ B α degradation. In addition, grape seed proanthocyanidin markedly repressed the activity of NF- κ B and promoted apoptosis of TSCC cells by regulating the stability and activity of Bax and downgrading the Bcl-2 and Bcl-xL proteins, and additionally suppressed the aggressiveness and metastasis of TSCC cells by restraining the secretion of MMP-2 and MMP-9 in the cells (68). A previous study reported that isobavachalcone may inhibit the expression of the apoptosis-related protein Bcl-2 (69).

Based on these previous studies and the decreased expression of Bcl-2, MYC and NF- κ Bp65 in the present study after EDARADD knockdown, the possible mechanism of action of EDARADD in TSCC can be hypothesized. It can be speculated that knockdown of EDARADD plays a role in the growth and survival of TSCC cells by affecting the NF- κ B and relevant pathway genes. Though a significant biological effect of EDARADD has been detected at the cellular level in *in vitro* studies, further *in vivo* experiments are necessary to confirm whether the EDARADD gene is a genuine target in TSCC and to explore its underlying molecular mechanism.

In summary, it is speculated that knockdown of EDARADD may influence the expression of NF- κ B and relevant pathway genes and thereby suppress cellular proliferation along with tumor cell apoptosis in TSCC. These findings suggest that EDARADD may serve as a novel therapeutic target and latent candidate for TSCC treatment.

Acknowledgements

Not applicable.

Funding

The present study was supported by the grants from Jiangsu Health and Family Planning Commission Project of Scientific Research (grant no. H2017080) and Xuzhou Science and Technology Project (grant no. KC17196).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The data generated by TCGA Research Network (<http://cancergenome.nih.gov/>) has been used for UALCAN development (<http://ualcan.path.uab.edu/>).

Authors' contributions

JM was involved in project administration and supervision, playing a guiding role in the entire experimental research process. ML and JM contributed to the conception of the investigation. ML and YTB carried out the experiments. KH and XDL were responsible for collecting experimental data and analyzing data and all authors explained and discussed the results. ML wrote the manuscript and YTB revised it. YTB contributed to supplementary experiments. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by The Ethical Committee of the Central Hospital of Xuzhou and the Xuzhou Clinical College of Xuzhou Medical University (reference no. 2009XL002) and written informed consent was obtained from each patient.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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