

Overexpression of tumor suppressor gene ZNF750 inhibits oral squamous cell carcinoma metastasis

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Abstract. Zinc-finger protein 750 (ZNF750) encodes a putative C2H2 zinc finger protein and is typically mutated or deleted in squamous cell carcinoma. The role of ZNF750 in oral squamous cell carcinoma (OSCC) remains unknown. The aim of the present study was to investigate the effects of ZNF750 overexpression in CAL-27 cells. Cell viability, and the expression of genes associated with proliferation, differentiation and the epithelial-mesenchymal transition were investigated in CAL-27 cells following ZNF750 overexpression, using Cell Counting kit-8, reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively. In addition, scratch wound, invasion and migration assays were performed. Cell viability, matrix metalloproteinase 28 expression, cyclin B1 expression and mesenchymal marker neural cadherin expression were decreased following ZNF750 overexpression compared with the control groups. ZNF750 overexpression induced the differentiation-associated genes late cornified envelope 3A and small proline-rich protein 1A and upregulated the expression of late epidermal differentiation factor Kruppel-like factor 4. Overexpression of ZNF750 in CAL-27 cells resulted in inhibition of cell invasion and

migration. Taken together, these data suggest that ZNF750 may inhibit the metastasis of OSCC.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is a common type of cancer and represents ~6% of all diagnosed cases of cancer (1). Oral squamous cell carcinoma (OSCC) is associated with a high rate of morbidity and mortality, with few therapeutic options (2). The survival rate for patients with OSCC has not improved in recent decades despite the development of novel therapies (3). It has been reported that >90% of cancer-associated mortalities are the result of metastasis (4). Understanding underlying molecular mechanisms involved in the metastasis of OSCC is therefore a priority (4).

Zinc-finger protein 750 (ZNF750) is a putative C2H2 zinc finger protein and is typically expressed in keratinocytes (5). It is an essential regulator of epidermal differentiation; it binds and activates the epidermal differentiation genes, including late cornified envelope 3A (LCE3A) and small proline-rich protein 1A (SPRR1a), and represses epidermal progenitor genes, including matrix metalloproteinase (MMP) 28 (6). Epidermal differentiation involves the repression of progenitor genes, which participate in cell proliferation and adhesion to the underlying basement membrane (7). A loss in cell-cell adhesion and an increase in cell motility are prerequisites for cancer metastasis (7).

ZNF750-repressed genes have previously been enriched for terms relevant to cell proliferation (6). Previous studies revealed that ZNF750 is typically mutated or deleted in squamous cell carcinoma (8,9). The loss of ZNF750 is associated with impaired differentiation and failure to fully repress the proliferative genetic program, both of which are key hallmarks of cancer (8). ZNF750-driven epidermal differentiation occurs partially through the induction of Kruppel-like factor 4 (KLF4), a transcription factor that activates late epidermal differentiation-associated genes and is critical for the epithelial-mesenchymal transition (EMT) (10,11). Deletion of KLF4 has been reported to be sufficient to initiate tongue carcinoma development (2).

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At present, the biological function of ZNF750 in the pathogenesis of OSCC is unknown. The present study aimed to investigate the effect of overexpressed ZNF750 on cell viability, invasion, migration and expression of EMT-associated genes in OSCC. Clarification of the role of ZNF750 in OSCC may contribute to the development of novel treatment strategies.

Materials and methods

Reagents. The lentiviral packaging plasmids psPax2, pRSV-Rev and VSV-G were provided by Dr Pdraig Strappe (Central Queensland University, North Rockhampton, Australia). The pLVX-PGK-Puro lentiviral vector backbone and the ZNF750 lentiviral vector pLVX-hZNF750-PGK-Puro were purchased from Biowit Technologies (Nanshan, China). Matrigel was obtained from BD Biosciences (Franklin Lakes, NJ, USA).

Cell culture and treatment. The OSCC cell line CAL-27 and 293T packaging cell line (purchased from the American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) (both from HyClone; GE Healthcare, Chicago, IL, USA) and 1% penicillin/streptomycin under standard cell culture conditions at 37°C with 5% CO₂. CAL-27 cells growing in the exponential phase were randomly divided into the following groups: Control, PGK (negative control, transduced with pLVX-PGK-Puro lentivirus) and ZNF750 groups (transduced with pLVX-hZNF750-PGK-Puro lentivirus).

Lentiviral packaging and CAL-27 cell transduction. Lentiviral vector packaging and transduction was performed as described previously with minor modifications (12). For the generation of LV-PGK and LV-ZNF750 lentivirus, 7 µg PGK or ZNF750 lentiviral vector plasmid together with packaging plasmids (7 µg psPax2, 3 µg pRSV-Rev and 3 µg VSV-G) were co-transfected into 70-80% confluent 293T cells, using Lipofectamine[®] 2000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Lipofectamine[®] 2000/DNA complexes were added to 293T cells in DMEM with 10% FBS and 1% penicillin/streptomycin to which caffeine (4 mM) and sodium butyrate (1 mM) were added to increase the lentiviral titer (13). At 48 and 72 h post-transfection, the virus particles present in the cell supernatant were harvested and centrifuged at 5,000 x g for 30 min to remove cell debris, then filtered through a Steriflip-HV 0.45 µm polyvinylidene fluoride (PVDF) filter unit (EMD Millipore, Billerica, MA, USA) and concentrated using PEG-it virus precipitation solution (System Biosciences, Inc., Palo Alto, CA, USA) to obtain virus particles. The CAL-27 cells were transduced with the LV-PGK or LV-ZNF750 lentiviral vectors (at a multiplicity of infection of 10) after the cells reached 60-70% confluence. The cells were allowed to recover for 48 h and puromycin (2 µg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added into the culture medium for stable cell line selection.

Cell viability analysis. Each group of cells was seeded in a 96-well plate at a density of 5x10⁴ cells/well. Cell viability following overexpression of ZNF750 was evaluated using a Cell

Counting Kit-8 (CCK-8; Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocol. The counting reagent (50 µl) was added to each well and incubated for 2 h. The absorbance was measured at 450 nm using a STAKMAX[™] microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA). Experiments were performed in triplicate at least three times.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT-qPCR was performed to examine expression of the epidermal progenitor genes MMP28 and the epidermal differentiation genes LCE3A and SPRR1a. According to the manufacturer's protocol, total RNA from cells was extracted with TRIzol reagent (Thermo Fisher Scientific, Inc.). RNA (1 µg) was converted to complementary DNA (cDNA) using a PrimeScript[®] RT kit (Takara Biotechnology Co., Ltd., Dalian, China), and SYBR[®] Green qPCR amplifications were performed in an ABI 7500 Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using AceQ qPCR SYBR[®] Green Master Mix (Vazyme, Piscataway, NJ, USA). The thermocycling conditions for the PCR were as follows: 95°C for 5 min followed by 40 cycles of 95°C for 10 sec and 57°C for 30 sec. The primer sequences were as follows: MMP28 forward, 5'-GAGCGTTTCAGTGGGTGTC-3' and reverse, 5'-CCATTTGTTACCTTGCTTTGC-3'; LCE3A forward, 5'-AGCACAGTGTCTGCCTCCA-3' and reverse, 5'-GGCATCTGTGGTGAAGTCAAG-3'; SPRR1a forward, 5'-AGCAGCAGCAGGTGAAACA-3' and reverse, 5'-GCTGGAGTGACCGTTGAAG-3'; GAPDH forward, 5'-TGCACCACCAACTGCTTAGC-3' and reverse, 5'-GGCATGGACTGTGGTTCATGAG-3'. The fold change amplification for MMP28, LCE3A and SPRR1a was normalized to the GAPDH housekeeping gene using the 2^{-ΔΔC_q} method (14). Each experiment was evaluated with three PCR reactions and each experiment was repeated three times.

Western blotting. Protein was extracted using radioimmunoprecipitation lysis buffer, including phenylmethylsulfonate fluoride, and the concentration was measured using a BCA protein assay kit (all from Beyotime Institute of Biotechnology). An equal amount of protein (15 µg) from each group were separated on a 10% SDS-PAGE gel and transferred onto PVDF membranes (EMD Millipore). The membranes were blocked using Tris-buffered saline with Tween-20 (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20) with 5% non-fat milk at 37°C for 1 h and probed with primary antibodies: Mouse monoclonal anti-cyclin B1 (cat no. 4135), rabbit polyclonal anti-neural (N)-cadherin (cat no. 4061) (both from Cell Signaling Technology, Inc., Danvers, MA, USA) and anti-KLF4 antibodies (cat no. ab106629; Abcam, Cambridge, UK) at a dilution of 1:1,000 overnight at 4°C. Following incubation with a goat anti-mouse or goat anti-rabbit immunoglobulin G/horseradish peroxidase conjugated secondary antibody (dilution, 1:1,000; cat no. AA128; Beyotime Biotechnology, Jiangsu, China) at 37°C for 1 h, membranes were visualized using an enhanced chemiluminescence reagent from Beyotime Institute of Biotechnology. Densitometry analysis was performed using AlphaView analysis software (AlphaMager[®] 2200; ProteinSimple; Bio-Techne, Minneapolis, MN, USA).

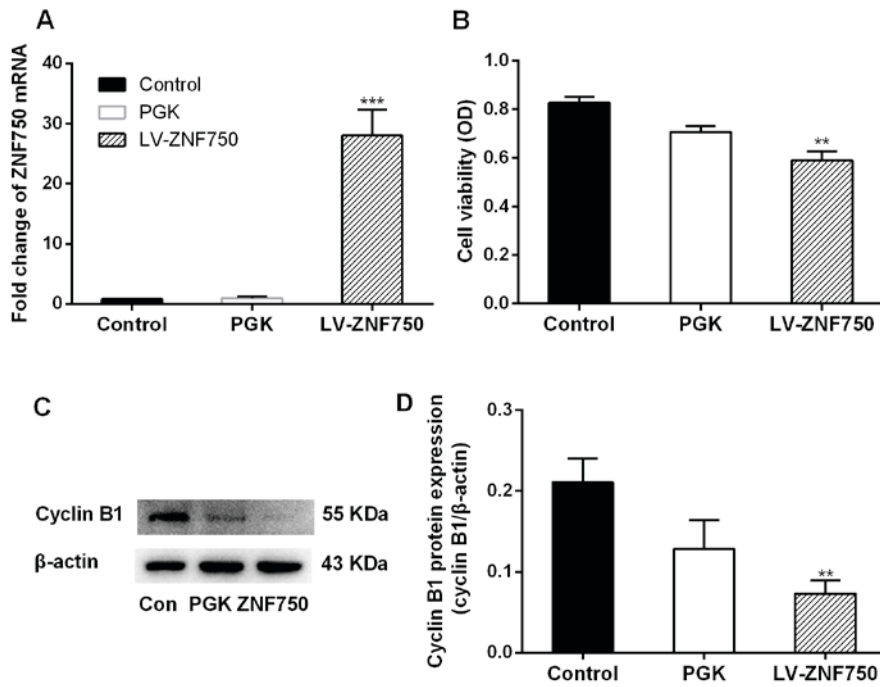


Figure 1. ZNF750 decreases CAL-27 cell viability. (A) ZNF750 mRNA expression (B) cell viability (C) cyclin B1 protein expression and (D) quantification of cyclin B1 protein expression in CAL-27 cells following overexpression of ZNF750. ** $P < 0.01$, *** $P < 0.001$ compared with the control group. Each experiment was repeated three times. ZNF750, zinc-finger protein 750; PGK, negative control transduced with pLVX-PGK-Puro lentivirus; LV-ZNF750, transduced with pLVX-hZNF750-PGK-Puro lentivirus.

Cell scratch, invasion and migration assay. Cells were seeded on 6-well plates and grown to 80% confluence. The cell monolayer was gently scraped with a sterile 200 μ l pipette tip and the wells were washed twice with PBS to remove the cell debris. The width of the scratch was determined by images taken under light microscopy at 0 and 24 h after creating the wound. The cells in three wells of each group were quantified. Three independent experiments were performed.

For the cell invasion assay, Corning Transwell® chambers with polycarbonate membrane (8 μ m pore size) were used to examine the effect of ZNF750 on CAL-27 cell invasion. Matrigel (BD Biosciences) was used as the substrate for invasion as previously described (15). Briefly, cells (1×10^5) were dispersed onto Matrigel-coated polycarbonate membranes at a dilution of 1:6 in serum-free culture medium in the upper chamber. DMEM containing 10% FBS was added to the lower chamber. Invasion was allowed to proceed for 24 h at 37°C in 5% CO₂. Cells remaining attached to the upper surfaces of the Matrigel-coated polycarbonate membrane (non-invading cells) were carefully removed with cotton swabs. Cells that had invaded to the lower surfaces of the membrane were fixed with 4% formaldehyde for 15 min, stained with 0.1% crystal violet for 10 min and visualized under a light microscope (CKX71; Olympus Corporation, Tokyo, Japan). Assays were performed in triplicate and the average number of invaded cells per field was assessed using Image-Pro® Plus 6 software (Media Cybernetics, Inc., Rockville, MD, USA). The cell migration assay was performed with a similar protocol to the invasion assay, but without a Matrigel coating.

Statistical analysis. Values are expressed as the mean \pm standard deviation. The data were analyzed by using a one-way analysis of variance followed by a Student-Newman-Keuls-q test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

ZNF750 decreases CAL-27 cell viability. ZNF750 mRNA expression was significantly increased (~28-fold) in the LV-ZNF750 group compared with the control and PGK groups ($P < 0.001$; Fig. 1A). In addition, a significant decrease in cell viability and cyclin B1 expression was observed following ZNF750 overexpression compared with the control groups (both $P < 0.01$; Fig. 1B and C).

ZNF750 induces the expression of differentiation-associated genes and reduces the expression of progenitor genes in CAL-27 cells. Following ZNF750 overexpression, MMP28 was significantly downregulated (~2-fold; $P < 0.001$; Fig. 2A); however, the expression of the differentiation genes LCE3A and SPRR1a was significantly increased (~5- and 3-fold, respectively; $P < 0.001$) compared with the control groups as measured by RT-qPCR (Fig. 2A). The expression of the terminal epidermal differentiation driver KLF4 was significantly increased at the mRNA and protein level following ZNF750 overexpression compared with the control groups ($P < 0.001$ and $P < 0.01$, respectively; Fig. 2). There was a 2-fold increase in KLF4 protein expression in the ZNF750 overexpression group compared with the control groups (Fig. 2B).

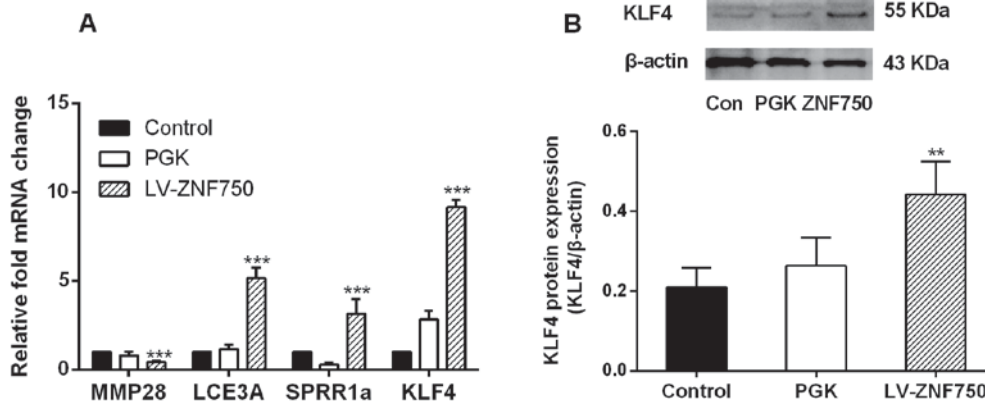


Figure 2. ZNF750 reduces the expression of the progenitor gene MMP28 but reduces the expression of differentiation-associated genes. (A) mRNA expression of MMP28, LCE3A, SPRR1a and KLF4 and (B) protein expression of KLF4 following overexpression of ZNF750. **P<0.01, ***P<0.001 compared with the control group. All experiments were performed in triplicate at least three times. ZNF750, zinc-finger protein 750; MMP28, matrix metalloproteinase; LCE3A, late cornified envelope 3A; SPRR1a, small proline rich protein 1A; KLF4, Kruppel-like factor 4. PGK, negative control transduced with pLVX-PGK-Puro lentivirus; LV-ZNF750, transduced with pLVX-hZNF750-PGK-Puro lentivirus.

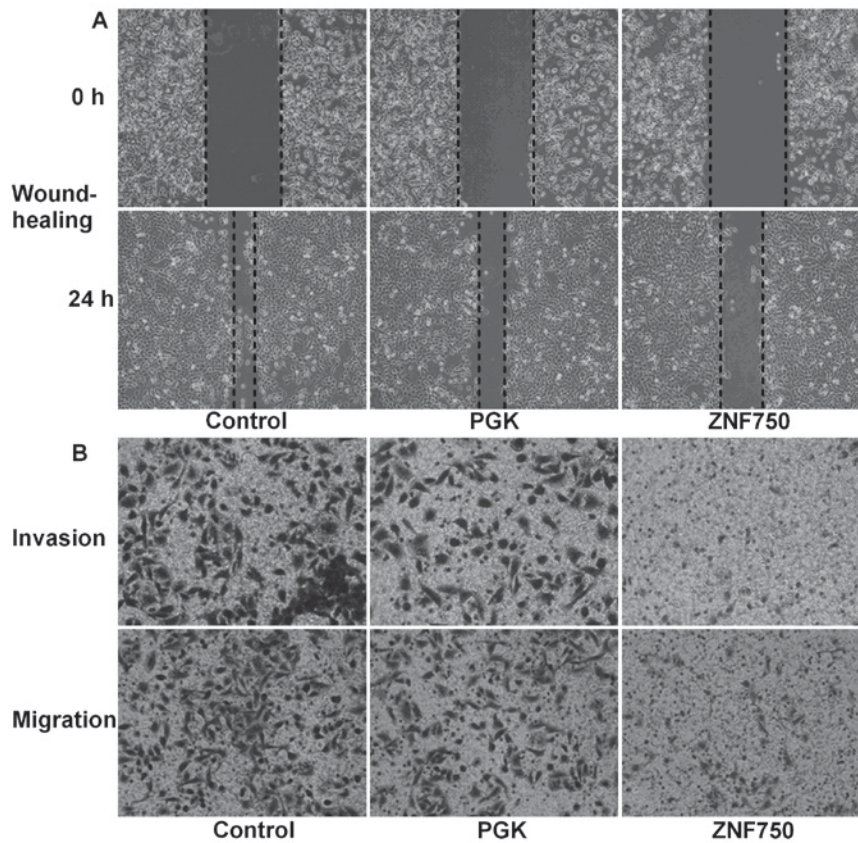


Figure 3. ZNF750 overexpression inhibits CAL-27 migration and invasion. (A) Scratch wound assay to analyze wound healing ability of CAL-27 cells following ZNF750 overexpression. (B) Cell invasion and migration assays following ZNF750 overexpression. ZNF750, zinc-finger protein 750; PGK, negative control transduced with pLVX-PGK-Puro lentivirus; LV-ZNF750, transduced with pLVX-hZNF750-PGK-Puro lentivirus.

ZNF750 overexpression inhibits cell migration and invasion. In order to estimate the metastatic ability of CAL-27 cells, cell invasion and migration were analyzed in addition to the expression of N-cadherin. A wound healing assay demonstrated that the migration distance covered by cells overexpressing ZNF750 was markedly decreased at 24 h (cells migrated only 25-30% into the scratch) compared with that covered by cells in the control and PGK groups, where

the cells had migrated 75-85% into the scratch (Fig. 3A). In addition, invasion and migration assays demonstrated that the number of cells that had migrated to the lower chamber in the ZNF750 group was markedly decreased compared with that of the control groups (Fig. 3B). Additionally, N-cadherin protein expression was significantly decreased in the ZNF750 group compared with the control group (P<0.01; Fig. 4).

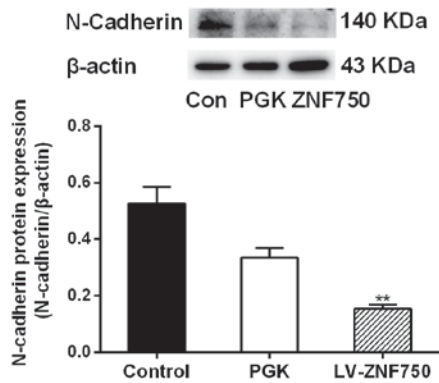


Figure 4. ZNF750 overexpression decreases the expression of the mesenchymal marker N-cadherin. N-cadherin protein expression was investigated by western blot analysis. ** $P < 0.01$ compared with the control group. PGK, negative control transduced with pLVX-PGK-Puro lentivirus; LV-ZNF750, transduced with pLVX-hZNF750-PGK-Puro lentivirus; con, control.

Discussion

ZNF750 is a gene that typically resides in a focal deletion in the HNSCC genome (16). Despite previous studies reporting the role of ZNF750 in epidermal differentiation (7,10,17), the role of ZNF750 in OSCC has not yet been documented. To understand the potential anticancer effects of ZNF750 on OSCC, the role of ZNF750 in cell proliferation, differentiation, invasion and migration was investigated. The data from the present study indicated that ZNF750 is a tumor suppressor gene in OSCC. ZNF750 overexpression inhibited CAL-27 invasion and migration, suggesting that ZNF750 may inhibit cell metastasis during cancer progression.

A previous study indicated that normal expression of ZNF750 leads to reduced expression of the progenitor gene MMP28, and increased expression of the differentiation genes LCE3A and SPRR1a mRNA in primary human undifferentiated keratinocytes (7). Loss of ZNF750 has been reported to result in impaired differentiation and enhanced proliferation of cancer cells (8). ZNF750 interacts with the terminal epidermal differentiation factor KLF4 to induce the expression of differentiation-associated genes (7), and KLF4 deletion is sufficient to impair squamous cell differentiation and initiate tongue carcinoma development (2). In line with these previous reports, the present study revealed that ZNF750 upregulates the differentiation genes LCE3A and SPRR1a, and increases the expression of KLF4, while reducing the expression of MMP28. Although KLF4 protein expression was not increased compared with its mRNA expression, KLF4 protein expression may promote the effects of ZNF750. The results from the present study demonstrated that the overexpression of ZNF750 in CAL-27 cells results in decreased cell viability and decreased expression of the cell cycle regulator cyclin B1. The cyclin B1 gene is maximally active during the G₂/M and M phases of the cell cycle. Downregulation of cyclin B1 induces G₂/M cell cycle arrest (18). Increased G₂/M arrest has been associated with enhanced apoptosis (19). The present study suggested that ZNF750 may be important for the control of cell-cycle phase progression and inhibition of cancer proliferation.

Data from the present study suggested that ZNF750 inhibits cancer cell metastasis. A wound healing assay demonstrated that the migratory capacity of CAL-27 cell was inhibited by ZNF750, confirming the anti-metastatic characteristics of ZNF750. In the control group, cells had migrated into 75-85% of the scratch at 24 h, while the ZNF750 overexpressing cells, migrated only 25-30% into the scratch following 24 h incubation. This assay may reflect the inhibited migratory activity of ZNF750-overexpressing cells. In addition, ZNF750 overexpression resulted in markedly decreased numbers of invaded/migrated cells compared with the control groups. ZNF750 overexpression resulted in decreased expression of the mesenchymal marker N-cadherin. N-cadherin expression is associated with the EMT phenotype and with enhanced invasion in cancer (20). N-cadherin serves an important role in the malignant behaviors of HNSCC (21). EMT is a crucial process involved in the initiation and progression of metastasis, regulating the detachment of cancer cells from the epithelium and facilitating their invasion into stromal tissues (22). A major hallmark of the EMT process is the loss or reduction of epithelial markers, including E-cadherin, accompanied by the gain of mesenchymal markers, including N-cadherin, a phenomenon known as 'cadherin switching' leading to the loss of cell-to-cell adhesion, promotion of invasion, and subsequent migration to distant regions (20). In the present study, N-cadherin protein expression was associated with the malignant behavior of OSCC cells. N-cadherin expression was decreased in ZNF750 groups compared with the control groups, suggesting that ZNF750 restrains the invasive potency of CAL-27 cells. The data from the present study suggest that ZNF750 inhibits the metastatic properties of CAL-27 cells.

In conclusion, the data from the present study indicate that ZNF750 induces anticancer/anti-metastatic effects by promoting cell differentiation-associated gene expression, and by inhibiting cell progenitor gene expression and the metastatic characteristics of CAL-27 cells. These findings highlight the role of ZNF750 as a tumor suppressor gene for OSCC. Further studies are required to investigate the potential of ZNF750 as a therapeutic target for cancer treatment.

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