KLF7 overexpression in human oral squamous cell carcinoma promotes migration and epithelial-mesenchymal transition

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Abstract. Krüppel-like factor 7 (KLF7) is a member of the KLF family of zinc finger transcription factors, and was the first KLF cloned using complementary DNA and polymerase chain reaction (PCR) techniques with human vascular endothelial cells as a template. In addition, KLF7 is known as the ubiquitous Krüppel-like factor, as it is widely expressed in numerous human tissues at low levels. In the present study, the function of KLF7 in migration and epithelial-mesenchymal transition (EMT), which are associated with tumor progression, was investigated in human oral squamous cell carcinoma (OSCC) cells. Genes that were differentially expressed in normal vs. OSCC tissue were identified in the Gene Expression Omnibus database, which identified upregulation of KLF7 in OSCC. The expression and subcellular location of KLF7 was then analyzed using immunohistochemistry. KLF7 expression was measured in three OSCC cell lines, and the two cell lines with the highest (HN13) and lowest (CAL27) KLF7 expression were selected for further analysis. Subsequently, HN13 cells with reduced KLF7 expression (sh-HN13) and CAL27 cells overexpressing KLF7 (OE-CAL27) were constructed. Transwell migration and wound healing assays were then used to analyze the migration of the cells. In addition, mRNA and protein expression levels of the EMT markers E-cadherin, N-cadherin, vimentin and snail were detected using reverse transcription-quantitative PCR and western blotting. KLF7 overexpression in OSCC was validated using tissue immunohistochemistry, which identified moderate to high cytoplasmic staining of KLF7 in OSCC cells. KLF7 knockdown and overexpression altered the migration ability of sh-HN13 and OE-CAL27 cells, which decreased and increased significantly respectively. Expression of E-cadherin, N-cadherin, vimentin and snail was markedly altered in sh-HN13 and OE-CAL27 cells, indicating changes in EMT status. The results of the present study suggest that KLF7 overexpression changes the migratory behavior of OSCC cells, and induces EMT and lymph node metastasis through the expression of snail.

Introduction

Oral squamous cell carcinoma (OSCC) is one of the most common types of cancer worldwide. The incidence of OSCC has remained fairly constant in the past, but now appears to have increased in certain parts of the world (1,2). Despite advances in prevention and treatment, the five-year survival rate following diagnosis remains low due to metastasis and local recurrence (3,4). A previous study revealed that certain genetic expression profiles in the primary tumor may predict the incidence of metastatic progression in OSCC (5). Thus, identification of the genetic and molecular factors underlying the metastatic process is crucial to increasing understanding of OSCC, and for insight into the development of therapeutic strategies to improve survival in patients with OSCC.

Krüppel-like factor 7 (KLF7), also referred to as ubiquitous Krüppel-like factor, is expressed at low levels in numerous human tissues (6). Previous studies have demonstrated that KLF7 regulates neural ectoderm and mesoderm lineage differentiation, and is important in the development of numerous other tissues and organs (7). KLF7 knockout mice exhibit phenotypes that suggest that KLF7 serves an important role in nervous system development (8). In addition, a previous study of embryonic stem cells showed that KLF7 serves a key role in the development of a number of tissues, including muscle and bone (7). Furthermore, studies in humans and mice have identified that KLF7 inhibits adipocyte differentiation and downregulates the expression of cellular differentiation markers (9). KLF7 modulates the expression of cytokine that are markers of human adipocyte differentiation and has a role in adipocyte maturation (10,11). In addition, a previous study in children with acute lymphoblastic leukemia has revealed that KLF7 overexpression inhibits the function of hematopoietic stem cells (12).

The KLF family is composed of individual KLF proteins with documented roles in carcinogenesis, including KLF4,

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5, 6, 8, 9, 10, 11 and 12. Among these, KLF4 and 5 have been the most extensively studied. KLF4 is expressed in the suprabasal layer of the esophageal squamous epithelium, while KLF5 is expressed in the basal layer. A previous study demonstrated that KLF4 and KLF5 promote proliferation, apoptosis and invasion in the human esophageal carcinoma cell line TE2 (13). However, few studies regarding the role of KLF7 in cancer have been published. Ciribilli *et al* (14) recently demonstrated that c-myc may inhibit KLF7 expression, but this association was weak. Thus, the role of KLF7 in cancer remains to be fully elucidated. To investigate the role of KLF7 in cancer, the present study conducted an analysis of differentially expressed genes between normal and OSCC tissues within the gene series (GSE)-30784 in the Gene Expression Omnibus (GEO) database (15). KLF7 was identified to be upregulated in OSCC tissue compared with normal tissue. Subsequent Gene Ontology function analysis revealed that KLF7 was associated with cell motility signaling pathways.

Epithelial-mesenchymal transition (EMT) is a pathological event associated with tumor progression, invasion and metastasis (16). EMT is a highly regulated process in normal embryonic development, during which cells switch from a polarized, immobile epithelial phenotype to a highly mobile fibroblastic or mesenchymal phenotype (17). EMT has been shown to serve an important role in the invasiveness of pancreatic cancer in human cell lines and animal models (18), in addition to models of OSCC, breast cancer and other cancer types (19,20). Therefore, in the present study, KLF7 was hypothesized to mediate EMT and promote tumor invasion in OSCC in the present study. To investigate this hypothesis, EMT and the migration ability of OSCC cells overexpressing KLF7 or with KLF7 knockdown was assessed.

Materials and methods

Differentially expressed genes. GSE-30784 was retrieved from the GEO database, and included data from 45 normal tissues and 167 oral squamous cell carcinoma tissues. Genes that were differentially expressed in the normal and oral squamous cell carcinoma sample groups were identified. Briefly, fold-change (tumor/normal) was first used to filter the differentially expressed genes. Only genes with a fold-change (tumor/normal) >1.5 were entered into the subsequent analyses. A random variance model *t*-test was then used to confirm the differentially expressed gene, as this test is able to effectively increase the degrees of freedom in small samples. Following analysis of the significance and the false discovery rate (FDR), genes for which P<0.05 and FDR<0.05 were considered to be significantly differentially expressed.

OSCC samples. OSCC tumor specimens used in the present study were obtained from the Department of Pathology at Zhongshan Hospital Affiliated to Fudan University (Shanghai, China) from 49 patients with a mean age of 43 years (standard deviation, 10), of whom 25 were male and 24 were female. All patients were first diagnosed with OSCC and had not undergone any treatment prior to visiting Zhongshan hospital between January 2006 and December 2006. Samples were stained with hematoxylin and eosin to analyze the

pathological differentiation and pathological diagnosis, which were confirmed by two experienced pathologists. The 49 pathological slides used in the present study included samples from 18 patients that had OSCC lymph node metastases. All patients gave informed consent for their samples to be used in the study. Zhongshan Hospital Ethics Committee approved the current study.

Immunohistochemistry. Formalin-fixed and paraffin-embedded 5-μm tissue sections were deparaffinized and rehydrated, followed by high-temperature antigen retrieval using a microwave in 0.1 M citrate solution (pH 6.0) for 15 min. Following blocking with 5% normal goat serum at room temperature for 30 min, the sections were incubated with rat anti-KLF7 antibody (#ab197690; 1:100, Abcam, Cambridge, UK) diluted in PBS containing 3% BSA (V900933, Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) at 4°C overnight, then incubated with horseradish peroxidase (HRP)-coupled secondary antibodies (cat. no. GK500705 Gene Tech, Shanghai, China), following the protocol of the ChemMate EnVision Detection kit, for 30 min at room temperature. 3,3'-Diaminobenzidine (DAB) reactions were performed using a ChemMate EnVision Detection kit (cat. no. GK500705; Gene Tech, Shanghai, China), Subsequently, the sections were stained with hematoxylin, washed with running water, dehydrated in a graded series of ethanol and xylene, and then mounted with a coverslip.

Two pathologists with no prior knowledge of the patients or their characteristics assessed the immunohistochemistry samples independently, where differences in opinion were resolved through reassessing samples. Immunoreactivity was assessed in a semi-quantitative manner through analyzing the extent and intensity of positively stained cells. Scores were assigned as follows: 0, ≤5% cells were positively stained; +1, between 6 and 20% of cells were positively stained; +2, between 21 and 50% of cells were positively stained; and +3, ≥50 % cells were positively stained. The total evaluation score was the product of the evaluations of the two pathologists, and the final scores were the difference between the evaluation score of the tumor and non-tumor tissues. Final scores >3.5 were considered to represent tumor-specific expression (18).

Cell culture and virus infection. Three human OSCC cell lines (CAL27, HN13 and HN30) and the normal oral keratinocyte cell line HOK were obtained from the Shanghai Key Laboratory of Stomatology (Shanghai, China). OSCC cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) and HOK cells were cultured in RPMI-1640 (both Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 1% glutamine and 1% penicillin-streptomycin. All cells were maintained in 5% CO_2 at 37°C. Prior to viral infection and following puromycin selection, KLF7 and HN13 cells were seeded at a density of 5x10^4 cells/well on 6-well plates overnight. Viral transfer vectors encoding green fluorescent protein (GFP) and human KLF7 short hairpin RNA (shRNA) or KLF7 were constructed by Shanghai Hanyi Co. (Shanghai,
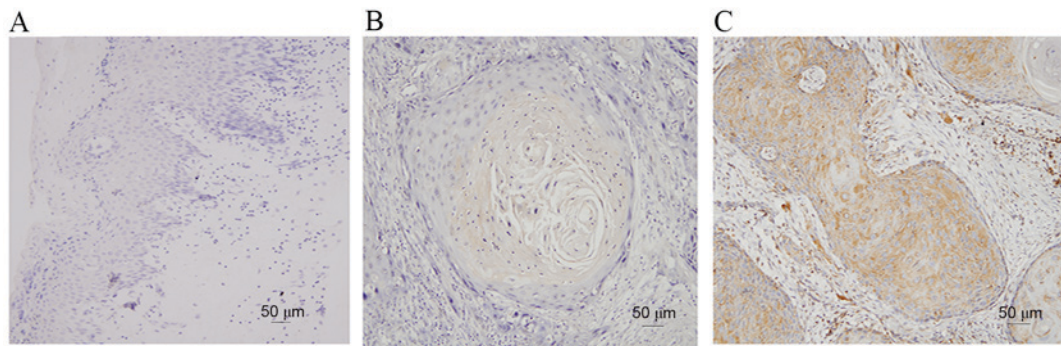
Figure 1. Representative images of KLF7 immunostained tissue samples. (A) Low level cytoplasmic expression of KLF7 in normal oral squamous mucosa. (B) Weak to moderate cytoplasmic expression of KLF7 in OSCC tissue. (C) High cytoplasmic expression of KLF7 in OSCC tissue. Magnification, x100.

China.) and transduced into HN13 and CAL27 cells, respectively. Stable clones were isolated by puromycin selection (1 μg/μl) over 4 weeks, generating an HN13 cell line stably expressing a KLF7 shRNA (sh-HN13) and a CAL27 cell line stably overexpressing KLF7 (OE-CAL27). Control cells were untreated cells, whereas negative controls (NC) consisted of cell infected with unvaccinated viruses (a group cell of viral vectors with no gene sequence inserted) were constructed by Shanghai Hanyi Co. (Shanghai, China).

Quantitative real time-polymerase chain reaction (RT-qPCR). Total RNA from all control, NC, and experimental groups of cells were extracted using TRIzol (Invitrogen, Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Reverse transcription was performed using PrimeScript RT Master Mix (Takara Bio, Inc., Otsu, Japan). RT-qPCR assays were performed using SYBR-Green Real-Time PCR Master mix (Takara Bio, Inc.) Human β-actin was used as an internal control to normalize the amount of mRNA in each sample using the $2^{-\Delta\Delta Cq}$ method (21). PCR thermocycling conditions were as follows: 1 cycle at 95°C for 30 sec; 40 cycles at 95°C for 5 sec; and 1 cycle at 60°C for 30 sec. All assays were performed in triplicate The primers used for the PCR reactions were: KLF7 forward (F), 5'-ACTGCTTGCTGACAATCT CG-3' and reverse (R), 5'-GGTCCCTCACACATCCTTCA-3'; E-cadherin F, 5'-GTCTCTCTCACCACCTCCACAG-3' and R, 5'-CTCGGACACTTCCACTCTCTTT-3'; N-cadherin F, 5'-TGCTACTTTCCTTGCTTCTGAC-3' and R, 5'-TAACAC TTGAGGGGCATTGTC-3'; vimentin F, 5'-GAAGAGAAC TTTGCCGTTGAAG and R, 5'-GAAGGTGACGAGCCA TTTC-3'; snail F, 5'-TCGGAAGCCTAACTACAGCGA-3' and R, 5'-AGATGAGCATTGGCAGCGAG-3'; and β-actin F, 5'-TGACGTGGACATCCGCAAAG-3' and R, 5'-CTGGAA GGTGGACAGCGAGG-3'.

Western blot analysis. Protein extracts from all control, NC, and experimental groups of cells were prepared using RIPA lysis buffer and protein concentrations were determined using the BCA Protein Assay kit (both Beyotime Institute of Biotechnology; Haimen, China). Samples (40 μg) were separated using SDS-PAGE on 10% gels and transferred onto polyvinylidene fluoride membranes. After incubation in 5% skim milk for 2 h at room temperature to block non-specific binding, the membrane was incubated for 16 h at 4°C with primary antibodies (all purchased from Abcam, Cambridge,

UK; dilution, 1:500) against KLF7 (#ab197690), E-cadherin (#ab15148), N-cadherin (#ab76057), vimentin (#ab137321), β-actin (#ab16039) and snail (#ab82846) diluted in PBS containing 3% BSA. The membrane was then washed 3 times with TBST (20 mM Tris-HCl, 137 mM NaCl and 0.1% Tween-20; pH 7.6) for 10 min/time, and incubated with anti-rabbit IgG-HRP antibodies (SC-2370, 1:5,000; Santa Cruz Biotechnology, Inc., Dallas, TX). Finally, the membrane was washed with TBST for 10 min 3 times. Following a further wash with TBST, signals were visualized using a Chemiscope5600 (Shanghai Clinx Science Instruments Co., Ltd., Shanghai, China) using MaxiLumin chemiluminescence detection reagents (Biokit, Barcelona, Spain).

Migration and wound healing assays. For transwell migration assays, 5x10⁵ sh-HN13 and OE-CAL27 cells were seeded into the upper chamber in 200 μl of serum-free medium, and 600 μl of DMEM supplemented with 10% FBS was added to the lower compartment. The cells were incubated at 37°C for 24 h, followed by fixation with methanol and cells that migrated into the lower chamber being stained with Giemsa solution (Sigma-Aldrich; Thermo Fisher Scientific, Inc.). To quantify the number of invading cells, five fields were randomly selected and images were captured using a light microscope for each transwell at a magnification of x100 and ImageJ software (1.48U; National Institutes of Health, Bethesda, MD, USA) was used to count the number of migrant cells in each image. For wound healing assays, 5x10⁵ sh-HN13 and OE-CAL27 cells were seeded in 6-well plates in their appropriate culture media, and wounds were made by scratching a line across the bottom of the dish on a monolayer of confluent cells with a 200 μl pipette tip. The gap between the cells at 0 and 24 h was imaged at a magnification of x100 on a microscope and then quantified using ImageJ software.

Statistical analysis. Statistical analysis of immunohistochemical staining was performed using SPSS software (version 19.0; IBM, Armonk, NY, USA). The Student's t-test and one-way analysis of variance were performed to compare the significance of differences between the two or three groups, respectively. All the experiments were performed in triplicate and, where possible, samples were processed twice. Results are presented as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Table I. Association between KLF7 and pathological characteristics of OSCCs in patients.

| Pathological characteristic | Number of patients | KLF7 protein expression level | | P-value |
		Low (number patients, %)	High (number of patients, %)	
Lymph nodes metastasis				0.002
Yes	18	2 (11.1)	16 (88.9)	
No	31	17 (54.8)	14 (45.2)	
Location				0.048
Epithelium	6	2 (33.3)	4 (66.7)	
Epithelial and mesenchymal	43	10 (23.7)	33 (76.7)	
Gender				0.506
Male	25	14 (56.0)	11 (44.0)	
Female	24	11 (45.8)	13 (54.2)	
Age (years)				1.009
>50	17	10 (58.8)	7 (41.2)	
≤50	32	14 (43.8)	18 (56.3)	

Total number of patient, 49. KLF7 protein expression levels determined by semi-quantification of immunohistochemistry staining. KL7, Krüppel-like factor 7.

Results

Expression of KLF7 protein in OSCC tissue. The expression of KLF7 protein in OSCC tissue was examined using immunohistochemistry. KLF7 protein was detected in all OSCC samples (49/49) with between moderate and high cytoplasmic staining. (score: +1,6-20%). However, only weak KLF7 staining was observed in the basal cells of the adjacent normal oral epithelium. All patients exhibited increased KLF7 expression compared with normal oral epithelium (Fig. 1). Furthermore, significantly more patients with OSCC with lymph nodes metastases had high levels of KLF7 protein expression compared with patients without lymph node metastases (score: +3, ≥50%; P=0.002; Table I). These results suggest that KLF7 expression is associated with pathological characteristics in OSCC. However, there was no significant association between the expression of KLF7 protein and the gender or age of the patients (Table I).

Modulating KLF7 expression. Among the OSCC cell lines tested, RT-qPCR demonstrated that KLF7 mRNA expression was highest in HN13 cells and lowest in CAL27 cells. These results were significant (*P<0.05 both CAL27 and HN13 vs. HOK group; Fig. 2A). Accordingly, HN13 and CAL27 cells were chosen for further analysis. Incorporation of KLF7 overexpression and KLF7 shRNA vectors was verified through GFP expression, which was 100% following Puromycin selection. mRNA and protein levels of KLF7 4 weeks following viral infection were determined by RT-qPCR and western blotting, respectively. mRNA expression levels of KLF7 significantly decreased in sh-HN13 cells compared with the NC group (***P<0.001, sh-HN13 vs. NC group; Fig. 2B), with a similar trend was seen for KLF7 protein expression (Fig. 2C). Conversely, KLF7 mRNA levels were significantly increased

in OE-CAL27 (***P<0.001 OE-CAL27 vs. NC group; Fig. 2D) and protein levels were notably increased (Fig. 2E).

KLF7 expression affects cell migration. To investigate the effect of KLF7 on the motility of OSCC cells, transwell and wound healing assays were performed. The results demonstrated that reducing KLF7 expression significantly repressed the migration capacity of sh-HN13 cells (**P<0.01 sh-HN13 vs. NC group; Fig. 3A and B, and that KLF7 overexpression significantly enhanced OE-CAL27 cell migration (*P<0.05 OE-CAL27 vs. NC group; Fig. 3C and D).

KLF7 modulates the expression of markers of EMT. As EMT is associated with the invasion and migration of cancer cells, the mRNA and protein expression levels of the EMT markers E-cadherin, N-cadherin and vimentin was investigated in sh-HN13 and OE-CAL27 cells was investigated using RT-qPCR (Fig. 4A and B) and western blotting (Fig. 4C), respectively. The mRNA level of E-cadherin in sh-HN13 cells was significantly increased compared with the NC group (*P<0.05 sh-HN13 vs. NC group; Fig. 4A) and a similar effect was seen for protein levels (Fig. 4C). However, in OE-CAL27 cells E-cadherin mRNA levels were significantly decreased compared with the NC group (**P<0.01 OE-CAL27 vs. NC group; Fig. 4B), with a similar effect seen in protein levels (Fig. 4C). In contrast, mRNA expression of the mesenchymal cell markers N-cadherin and vimentin significantly decreased in sh-HN13 cells (*P<0.05 sh-HN13 vs. NC group; Fig. 4A), but significantly increased in OE-CAL27 cells (*P<0.05 OE-CAL27 vs. NC group; Fig. 4B).

In addition, the expression of the transcription factor snail was measured to determine whether it was involved in KLF7-induced EMT in OSCC cells. Snail mRNA expression was significantly reduced in sh-HN13 cells (*P<0.05 sh-HN13
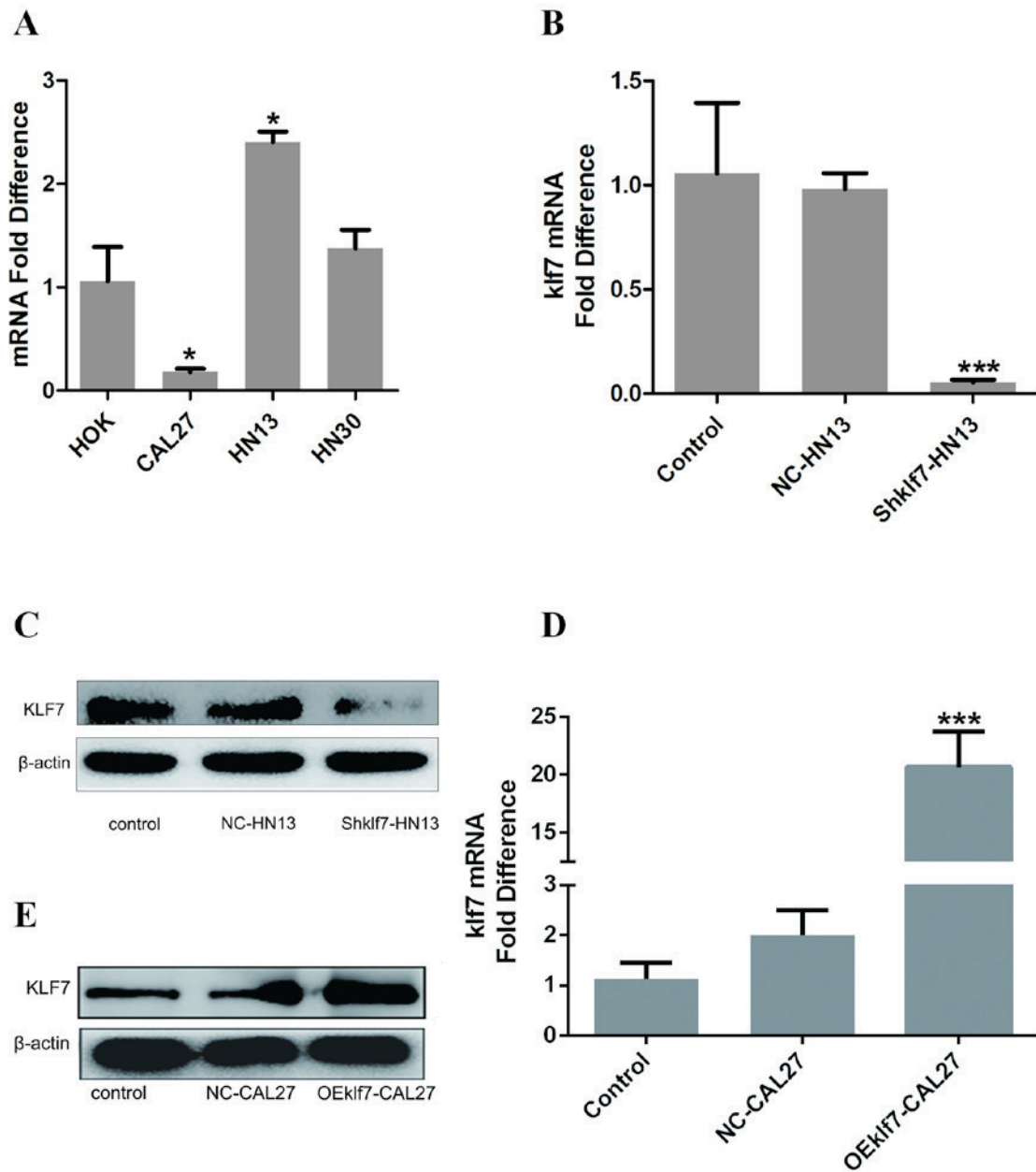
Figure 2. KLF7 expression following viral transduction. (A) KLF7 mRNA expression in three human OSCC cell lines (CAL27, HN13 and HN30) and one normal oral keratinocyte cell line HOK, (*P<0.05 CAL27, HN13 vs. HOK cells). (B) KLF7 mRNA expression in sh-HN13 cells was significantly decreased compared with the NC cells (***P<0.001 sh-HN13 vs. NC group). (C) Western blot analysis confirmed that expression of KLF7 protein in sh-HN13 cells was reduced. (D) KLF7 mRNA expression in OE-CAL27 cells was significantly increased compared with the NC cells (***P<0.001 OE-CAL27 vs. NC group). (E) Western blot analysis confirmed that the KLF7 protein levels in OE-CAL27 were increased. KLF7, Krüppel-like factor 7; NC, negative control; sh, shRNA; OE, overexpression.

vs. NC group; Fig. 5A) and increased in OE-CAL27 cells (*P<0.05 OE-CAL27 vs. NC group; Fig. 5B). Similar effects were seen in snail protein expression (Fig. 5C). No significant differences in Snail mRNA expression were observed between NC and control cells.

Discussion

In the present study, bioinformatic analysis of transcriptome data revealed that KLF7 was differentially expressed in OSCC compared with normal oral mucosa, while gene function analysis found that KLF7 expression was associated with cell motility. Immunohistochemistry confirmed that KLF7 protein

expression was increased in OSCC tissue vs. normal tissue, in addition to identifying that high expression levels of KLF7 were associated with lymph node metastases in patients with OSCC.

To further investigate the function of KLF7 in OSCC, the expression of KLF7 mRNA was measured in three OSCC cell lines, which identified that the expression was highest in HN13 cells and lowest in CAL27 cells. Subsequently, KLF7 expression was reduced or increased in these two cell lines, in order to study the effect of KLF7 on cell motility-related phenotypes, such as invasion and migration. Significantly less sh-HN13 and significantly more OE-CAL27 cells traveled through the transwell filter compared with the corresponding
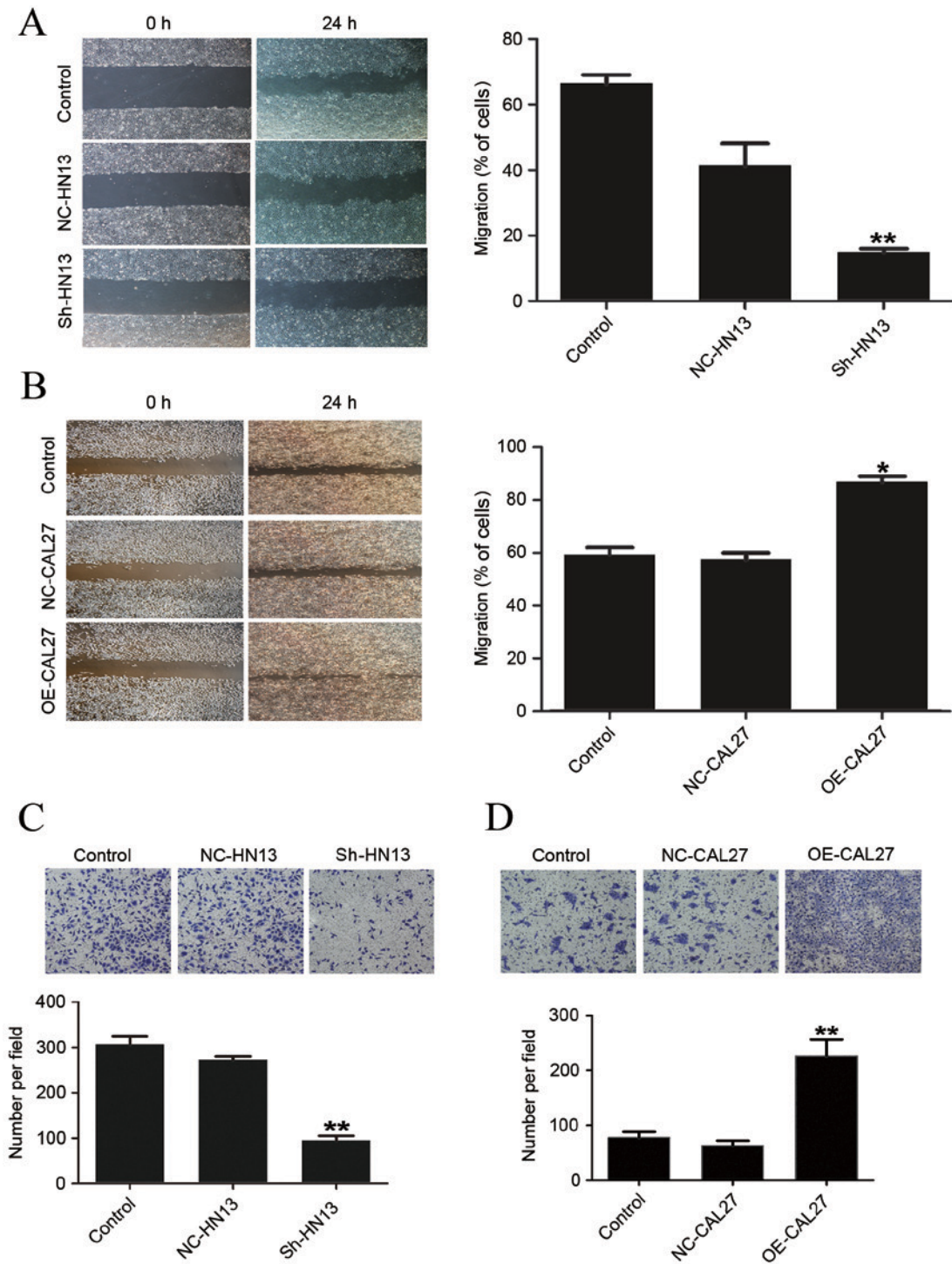
Figure 3. KLF7 expression affects the migration of OSCC cell lines. Representative images and quantification of wound healing assays with (A) sh-HN13 and (C) OE-CAL27 cells. Magnification, x40. Each value represents the mean ± the standard deviation of three experiments. Representative images and quantification of transwell assays with (B) sh-HN13 and (D) OE-CAL27 cells. Significantly fewer sh-HN13 and significantly more OE-CAL27 cells passed through the membrane of the chamber compared to NC cells. (**P<0.01 sh-HN13 vs. NC group; Fig. 3B), (*P<0.05 OE-CAL27 vs. NC group; Fig. 3D).

control cells. These results indicate that KLF7 serves a role in the invasion of OSCC cells.

Tumor cell invasion and migration occurs along with EMT, which involves the loss of epithelial cell markers and the expression of mesenchymal cell markers. This association has been documented during the process of tumor invasion in a number of solid tumors (22-26). EMT allows cells to be released from the primary tumor, invade adjacent tissues, enter the circulation and seed in distant organs. These changes in cell morphology and behavior following EMT are accompanied with alterations in gene expression (25). Following EMT, cells show a mesenchymal phenotype, including increased migration, invasion, apoptosis and chemo resistance. EMT is completed once the underlying basement membrane has been degraded, allowing interstitial cells to migrate from their origin to the rest of the body (27,28).
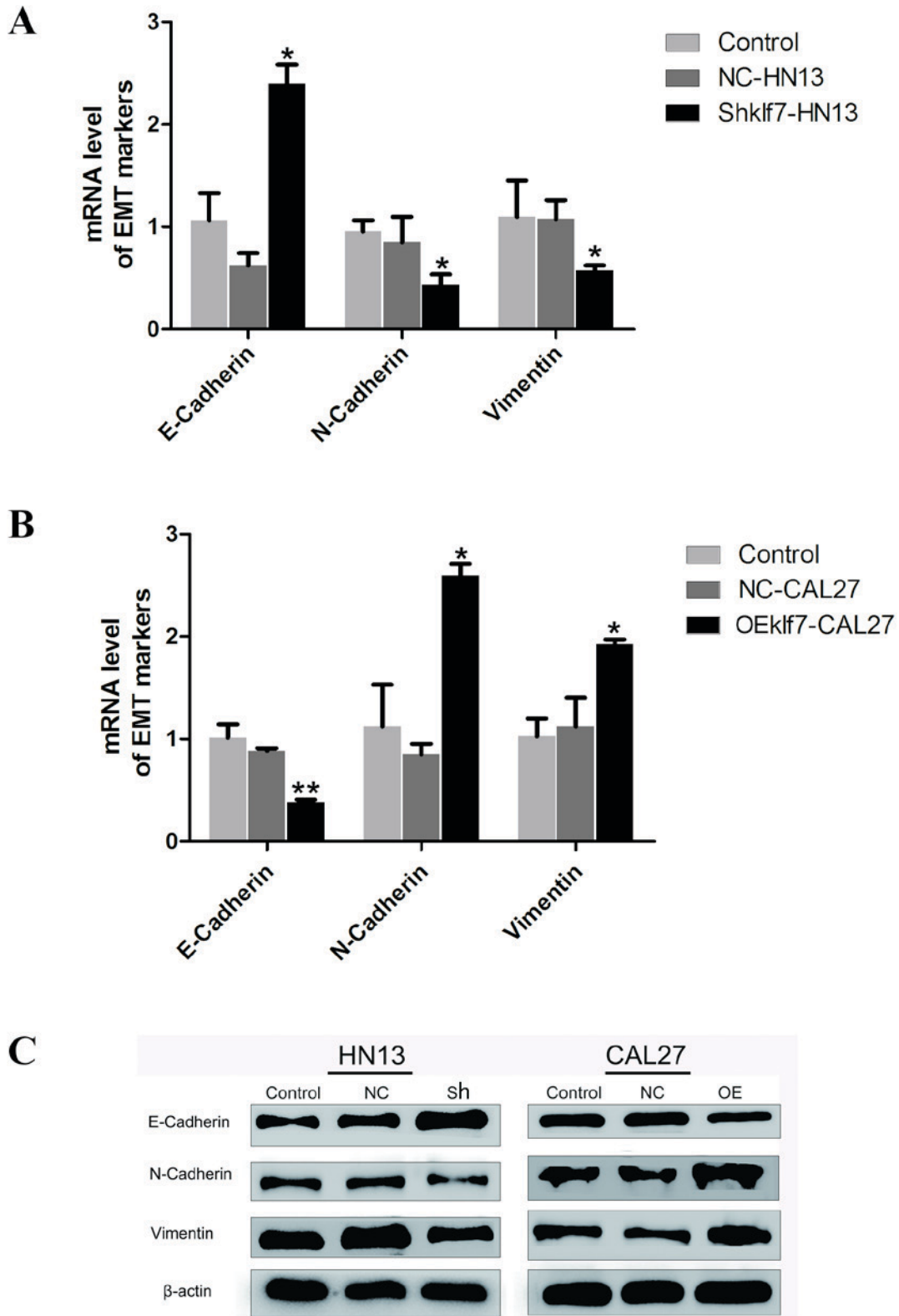
Figure 4. Effect of KLF7 on E-cadherin, N-cadherin and vimentin mRNA and protein expression. (A) Higher E-cadherin mRNA levels, and lower N-cadherin and vimentin mRNA levels were observed in sh-HN13 cells. (B) Lower E-cadherin mRNA levels, and higher N-cadherin and vimentin mRNA levels were observed in OE-CAL27 cells. (C) Western blot analysis of E-cadherin, N-cadherin and Vimentin protein levels in HN13 and CAL27 cells (E-cadherin: *P<0.05 sh-HN13 vs. NC group; **P<0.01 OE-CAL27 vs. NC group) (N-cadherin and Vimentin: *P<0.05 sh-HN13 and OE-CAL27 vs. NC group).

Compared with the NC, sh-HN13 cells had reduced mRNA and protein expression of vimentin and N-cadherin, and increased expression of E-cadherin. Conversely, the expression of N-cadherin and vimentin was increased, and

the expression of E-cadherin decreased, in OE-CAL27 cells compared with the NC. In addition, the present study identified that mRNA and protein expression snail was decreased in sh-HN13 cells compared with the NC. Snail, a zinc
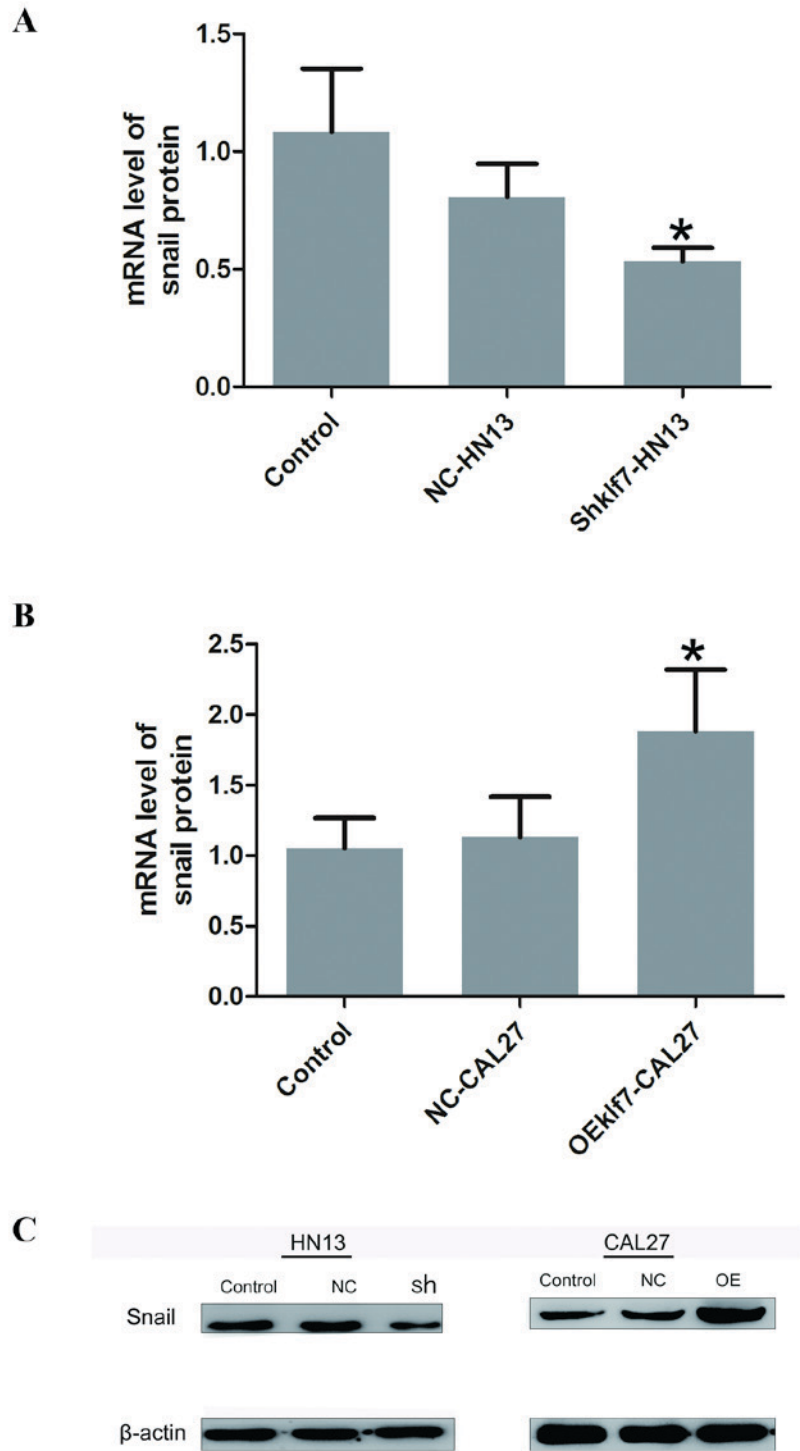
Figure 5. Effect of KLF7 on snail expression. (A) Lower snail mRNA levels were observed in sh-HN13 cells. (B) Higher snail mRNA levels were observed in OE-CAL27 cells. (C) Similar results were found for snail protein expression through western blot analysis (*P<0.05 sh-HN13 and OE-CAL27 vs. NC group).

finger-containing transcription factor, is a member of a family of transcription factors that repress expression of the adhesion molecule E-cadherin to regulate EMT during embryonic development (29). These results suggest that inhibition of KLF7 expression could inhibit or reverse EMT in OSCC.

When interpreting the results of the present study the following limitation should be considered. The results of the current study indicate that knockdown of KLF7 expression

inhibits migration and EMT in OSCC cells; however, the downstream signaling pathways that underlie the effects of KLF7 on EMT in OSCC have not yet been adequately investigated.

In conclusion, the results of the present study indicate that KLF7 serves an important role in the migration of OSCC cells. In addition, this highlights that reducing KLF7 expression may inhibit the migration of OSCC cells through blocking and

reversing EMT via the expression of snail. Thus, KLF7 may be a potential target for gene therapy to treat OSCC in the future and the specific molecular mechanisms underlying this activity of KLF7 in OSCC require further study.

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