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Original Article

CLIC1 promotes the progression of oral squamous cell carcinoma via integrins/ERK pathways

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Abstract: Chloride intracellular channel 1 (CLIC1), a member of the chloride channel protein family, acts as a promoter in many malignancies, but its role in oral cancer remains unclear. Hence, this research aimed to explore the effects of CLIC1 on the progression of oral cancer cells in vitro, and we assessed its role in cell proliferation, apoptosis, migration, invasion, angiogenesis, and chemosensitivity to cisplatin and possible signaling pathways. The results demonstrated that CLIC1 depletion inhibited the proliferation, invasion, migration and angiogenesis of oral squamous cell carcinoma (OSCC) cells in vitro, but promoted cell apoptosis and increased the drug susceptibility to cisplatin. In contrast, CLIC1 upregulation was positively correlated with cell proliferation, invasion and migration and angiogenesis. Mechanistically, CLIC1 silencing decreased the levels of ITGαv, ITGβ1, p-ERK, vimentin, MMP2 and MMP9, and increased the levels of p-p38, E-cadherin, caspase3 and caspase9. CLIC1 overexpression enhanced the ITGαv, ITGβ1, p-ERK, vimentin, MMP2 and MMP9 levels and decreased E-cadherin expression. Overall, these results indicated that CLIC1 promotes the progression of OSCC, and we speculated that its potential mechanism may be related to the regulation of ITGαv and ITGβ1, which led to activation of the MAPK/ERK and MAPK/p38 signal pathways.

Keywords: CLIC1, OSCC, Integrin, apoptosis, migration, pathways

Introduction

Oral cancer, including tongue cancer, gingival cancer, carcinoma in the floor of the mouth, and cancer of the jaw, is one of the most common malignant tumors of the head and neck. Oral squamous cell carcinoma is the maior pathological type and accounts for 90% of oral cancer cases [1, 2]. In recent years, the morbidity and mortality of oral cancer have gradually increased worldwide. There were more than 300,000 new cases and almost 200,000 deaths in 2018, and the five-year survival rate of oral cancer has been consistently lower than 50% in recent years [3-5]. Early oral cancer (stages I and II) can be cured by surgery or radiotherapy, but it is difficult to obtain satisfactory results for advanced cancer (stages III and IV), even with the combined treatment. Some approaches, such as targeted therapy, immunotherapy, and radioactive seed implantation, have not been fully developed [6]. Associations between the occurrence and development of oral cancer and genetic or epigenetic abnormalities have been reported [6, 7]. Thus, it is essential to study the molecular mechanisms of oral cancer progression to identify useful biomarkers that could be utilized for the improvement of clinical diagnosis and treatment.

Chloride intracellular channel 1 (CLIC1) is an ion channel protein that belongs to the CLIC family. CLIC1 is widely distributed and can be detected in many tissues from various species, such as rat, rabbit, normal human heart, liver, kidney, blood vessels and several tumor tissues [8]. Recent studies have shown that CLIC1 is involved in the regulation of cell cycle, apoptosis, osteogenesis, platelet release, and nervous system development [9, 10]. Another report showed that high tumor cell proliferation,

Table 1. Quantitative real-time PCR primers used in this study

Gene	Primer sequence (Forward)	Primer sequence (Reverse)
CLIC1	AATCAAACCCAGCACTCAATG	CAGCACTGGTTTCATCCACTT
GAPDH	CAACGACCCCTTCATTGACC	CGCCAGTAGACTCCACGACAT
MMP2	GCGCCGTCGCCCATCATCAA	AGCTCTCCTTGGGGCAGCCA
MMP9	GGGACGCAGACATCGTCATC	TCGTCATCGTCGAAATGGGC
Caspas3	ATGGACAACAACGAAACCTCCGTG	CCACTCCCAGTCATTCCTTTAGTG
Caspas9	GGCCCTTCCTCGCTTCATCTC	GGTCCTTGGGCCTTCCTGGTAT

active migration and invasion to nontumor tissues required some or even all of the chloride channels, and increasing evidence has demonstrated that chloride channels play an important role in the development of cancers [11]. As an important member of the CLIC family, CLIC1 has been studied in several malignancies, such as hepatocellular carcinoma, gastric cancer, esophageal cancer, choriocarcinoma, gallbladder cancer, colon cancer and neurologic tumors [12-17], but the relationship between CLIC1 and oral cancer remains unclear.

Previous results from our group showed that CLIC1 was highly expressed in OSCC tissues and plasma of patients, and high CLIC1 expression was distinctly associated with histological grade, TNM stage, tumor size and overall survival rate [18]. To further elucidate the relationship between CLIC1 and OSCC, we aimed to investigate the effects of CLIC1 on the biological behaviors of OSCC cells in vitro and performed a preliminary study of its potential molecular mechanisms.

Materials and methods

Cell culture

SCC-15 cells (ATCC, USA) were incubated on DMEM/F12 medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (NTC, Cordoba, Argentina) and 1% penicillin/streptomycin (HyClone, Logan, UT, USA) at 37°C and 5% CO_2 in a humidified incubator. Cells in the logarithmic phase were utilized in further studies.

Establishment of stably transfected OSCC cell lines

The lentiviruses included Lv-CLIC1 (CLIC1-overexpressing lentivirus), Lv-CLIC1-RNAi (CLIC1-RNA interference lentivirus) and Lv-shNC (blank lentivirus) plasmids, which were designed and generated by GENECHEM (Shanghai, China). According to the manufacturer's instruction, we obtained the appropriate MOI values (MOI = virus titer × virus volume/number of cells) and infection conditions for SCC-15 cell lines in the pilot experiment. Then, the lentiviruses were used to infect the SCC-15 cells, and puromycin was applied to collect single clones showing infection efficiency ≥80% and good growth status by microscopic observation. Finally, we obtained stable OSCC cell lines with CLIC1 knockdown (CLIC1-KD), CLIC1 overexpression (CLIC1-OE) and CLIC1 shNC (NC), and these cells were analyzed in the following experiments.

Reverse transcription polymerase chain reaction

Total RNA was extracted by using an RNA plus kit (Takara, Kusatsu, Japan). After quantification by a NanoDrop 2000 spectrophotometer (Thermo, USA), the cDNA was generated by using a Bio-Rad T100 Thermal Cycle (Bio-Rad Laboratories, Hercules, CA, USA) and SYBR Premix Ex Taq II (Takara, Japan). The specific primers used in this experiment were synthesized by TaKaRa Bio, and the sequences are displayed in **Table 1**. The total reaction volume was 20 μl. GAPDH was used as an internal control. The PCR condition included 95°C for 30 s, followed by 40 cycles of two steps: 95°C for 5 s and 60°C for 30 s and then 95°C for 5 s and 60°C for 30 s. The fl0°C for 30 signals were collected between 65°C and 95°C for melting curve analysis. The relative quantification of genes was calculated by using the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Cells or fresh tissues were lysed in the RIPA buffer (Beyotime, Shanghai, China) containing protease inhibitor cocktail (Beyotime, Shanghai, China). The protein concentration was measured by a BCA Protein Quantitative Kit (Beyotime, Shanghai, China). Separation gels and stacking gels (GenStar, Beijing, China) were fabricated. Proteins were separated by 6%, 10% or 15% sodium dodecyl sulfate (SDS)-

polyacrylamide gel electrophoresis (PAGE) and were transferred onto a PVDF membrane. The membrane was blocked with 5% BSA in TBST for 1 h and then incubated with primary antibody overnight at 4°C. The primary antibodies were as follows: CLIC1 (1:1000), ERK1/2 (1:1000), p38 (1:1000), caspase3 (1:1000), caspase9 (1:1000), ITGαv (1:5000) and ITGβ1 (1:2000), which were purchased from Abcam (Cambridge, MA, UK). Phospho-ERK1/2 (1:5000), phospho-p38 (1:5000), vimentin (1:1000), and E-cadherin (1:1000) were from CST (Danvers, MA, USA). β-actin (1:5000), MMP2 (1:500), MMP9 (1:500), and MMP13 (1:500) were obtained from ZENBIO (Chengdu, China). The membrane was incubated with the secondary antibody (1:8000, ZENBIO, Chengdu, China) for 1 h at room temperature. The immunoreactivity signals were developed after adding ECL reagent (Beyotime, Shanghai, China) and recorded by a Bio-Rad GelDoc 2000 Gel Imaging System (Bio-Rad Laboratories, Hercules, CA, USA). The gray values of protein bands were quantified by using ImageJ analysis software (Media Cybernetics, Inc.).

Cell Counting Kit-8 assay

The cell viability and drug susceptibility were tested by Cell Counting Kit-8 (CCK-8, Beyotime, Shanghai, China). In the cell viability assay, cells were seeded into 96-well plates (3×10^3/well). At 6, 24, 48 and 72 h, the cells were incubated with 10 μl CCK-8 solution, and the absorbance (A) values at 450 nm were measured by a microplate reader (PORTON, Chongqing, China). The cell viability was measured with the following formula: $[(A_{\text{experimental group}} - A_{\text{blank group}})/(A_{\text{control group}} - A_{\text{blank group}})]$. The drug susceptibility assay was performed as described above except the cell density was 6×10^4/ml, and the cells were cultured with cisplatin for 24 h before detection.

Cell apoptosis analysis by flow cytometry

The apoptosis rate was measured with Alexa Fluor 647/PI double staining (Bioss, Beijing, China). The cell samples were gently washed twice with PBS and resuspended in 1 × binding buffer with a density of 1×10^6/ml. Then, 5 μl Alexa Fluor 647 solution and 10 μl propidium iodide staining solution were mixed with the cell suspension. After incubation without light for 15 min at room temperature, the cell apoptosis rate was analyzed using flow cytometry (FCM, BD Bioscience, San Jose, CA, USA).

Colony formation assay

Four group of cells were separated into individual cells with F12 medium containing 10% FBS and seeded in 6-well plates (2000/well), and the medium was replaced every 3 days. After 14 days, the clones were visible, and then, the cells were fixed with methanol and stained with 1% crystal violet. The clones were counted under a microscope.

Tube formation assay

Tube formation assays were performed to evaluated the influence of CLIC1 on angiogenesis in OSCC. Matrigel (BD Biosciences, San Jose, CA, USA) was melted in a 4°C refrigerator in advance, and then, the μ-Slide Angiogenesis (ibidi, Martinsried, Germany) as coated with 10 μl Matrigel and placed in a cell incubator for 40 min. HUVECs (3×10^5/ml) were cultured in conditioned medium from the supernatants of the four group of cells. Images were periodically collected, and the relative tubule length, number of tubules, and number of branch points were measured using Image-Pro Plus software (Media Cybernetics, Inc.).

Wound healing assay

Wound healing assays were performed to measure cell motility. Cells with a density of 5×10^5/ml were seeded into a 6-well plate and incubated for 24 h, and then, complete medium was replaced with serum-free medium after drawing parallel lines in the plates. Wound closure was viewed under the microscope at 0 h, 24 h, and 48 h. ImageJ software (Media Cybernetics, Inc.) was used to measure the wound width.

Cell migration and invasion assays

Cell migration and invasion assays were performed by the Transwell method (8-μm pore size; HyClone, UT, USA). The cells were seeded into the upper chamber with serum-free medium and cultured for 48 h. However, in the invasion assay, the upper chamber was coated with Matrigel matrix (3.5 mg/ml) (Solarbio, Beijing, China) before cells were seeded. Medium supplemented with 15% fetal bovine serum was added to the lower chamber. Then, the cells on

the upper surface were transferred to the lower surface. After 48 h, the cells remaining on the upper membrane were removed using a cotton swab, and cells on the lower surface were fixed with 4% paraformaldehyde solution (Mengbio, Chongqing, China) and stained with 1% crystal violet (Mengbio, Chongqing, China). Five microscopic fields were randomly selected, and the stained cells were counted.

Indirect immunofluorescence assay (IFA)

IFA was performed to observe the localization of CLIC1 in cells. The cells were seeded on cell slides (Solarbio, Beijing, China), which were placed in a 6-well plate. After incubation for 24 h, the cells on the slides were fixed and reacted with Triton X-100 (Beyotime, Shanghai, China), blocked with goat serum (Bioss, Beijing, China) and incubated with the primary antibody against CLIC1 at 4°C overnight. Next, the cells were incubated with the secondary antibody and stained with DAPI (Bioss, Beijing, China). The CLIC1 subcellular localization was detected by a confocal laser scanning microscope (Leica TCS SP8 MP, Germany).

In vivo tumor growth assay

Female BALB/C nude mice (5 weeks old, weighing approximately 18-20 g) were obtained from the Chongqing Medical University Experimental Animal Center. Mice were fed in an animal facility with a temperature at 26°C-28°C, 40%-60% relative humidity and a 12-h light/dark cycle. Cells (1.5×10^7) were injected subcutaneously into the dorsal area, and the tumor volume was measured by caliper once a week. The tumor volume was calculated as follows: 0.5 × length × width^2. Four weeks later, the mice were sacrificed, and the subcutaneous tumors, livers and kidneys were sliced, weighed and photographed. Then, half of the samples were soaked in 4% formaldehyde for hematoxylineosin staining and immunohistochemistry staining, and the remaining samples were analyzed by western blotting. Permission for the animal experiments was obtained from the Biomedical Ethics Committee of Chongqing Medical University.

Statistical analysis

All experiments were repeated at least three times independently, and each experimental group had three parallel groups. The data are presented as the mean ± standard deviation (SD). The differences between groups were analyzed using Student's t-test or one-way ANOVA and the Bonferroni post hoc test. The significance level was defined at $P<0.05$. Statistical analyses and plotting were performed using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 6.01 software (La Jolla, CA, USA).

Results

CLIC1 knockdown and CLIC1-overexpressing OSCC cell lines were established

Western blotting and RT-PCR analyses were performed to detect the CLIC1 expression after transfection, and the subcellular expression of CLIC1 was analyzed by IFAs. As shown in **Figure 1A-H**, the proportion of transfected cells carring GFP was greater than 80%. The CLIC1 protein and mRNA levels in the CLIC1 knockdown group were significantly lower than those in the control and shNC groups ($P<0.001$), while those in the CLIC1-overexpressing group were obviously higher ($P<0.01$) (**Figure 1U** and **1V**). IFAs were performed to determine the subcellular localization of CLIC1, and the results showed that it was observed in the nucleus, nuclear membrane, cytoplasm, and cell membrane. In addition, the red fluorescence in the CLIC1 knockdown group was weaker than that of the control and CLIC1-ovexpressing groups, especially in the nucleus. The red fluorescence in the CLIC1-overexpressing group was enhanced (**Figure 1I-T**). These results indicated that the stable CLIC1 knockdown and CLIC1-overexpressing OSCC cell lines were established successfully.

CLIC1 promotes OSCC cell viability and proliferation and regulates apoptosis in vitro

CCK-8 assays showed that the cell viability of CLIC1 knockdown cells notably decreased ($P< 0.0001$), while the cell viability of the CLIC1-overexprssing group was significantly increased ($P<0.0001$), compared with those of the control and shNC groups (**Figure 2A**). Moreover, fewer and smaller colonies were observed in the CLIC1 knockdown group compared with the control, shNC and CLIC1-overexpressing groups ($P<0.01$), while the number of colonies in the CLIC1-overexpressing group was the highest ($P<0.05$) (**Figure 2B**). The cell apoptosis rate was measured by FCM. The apoptosis rate of CLIC1-knockdown cells was much greater than

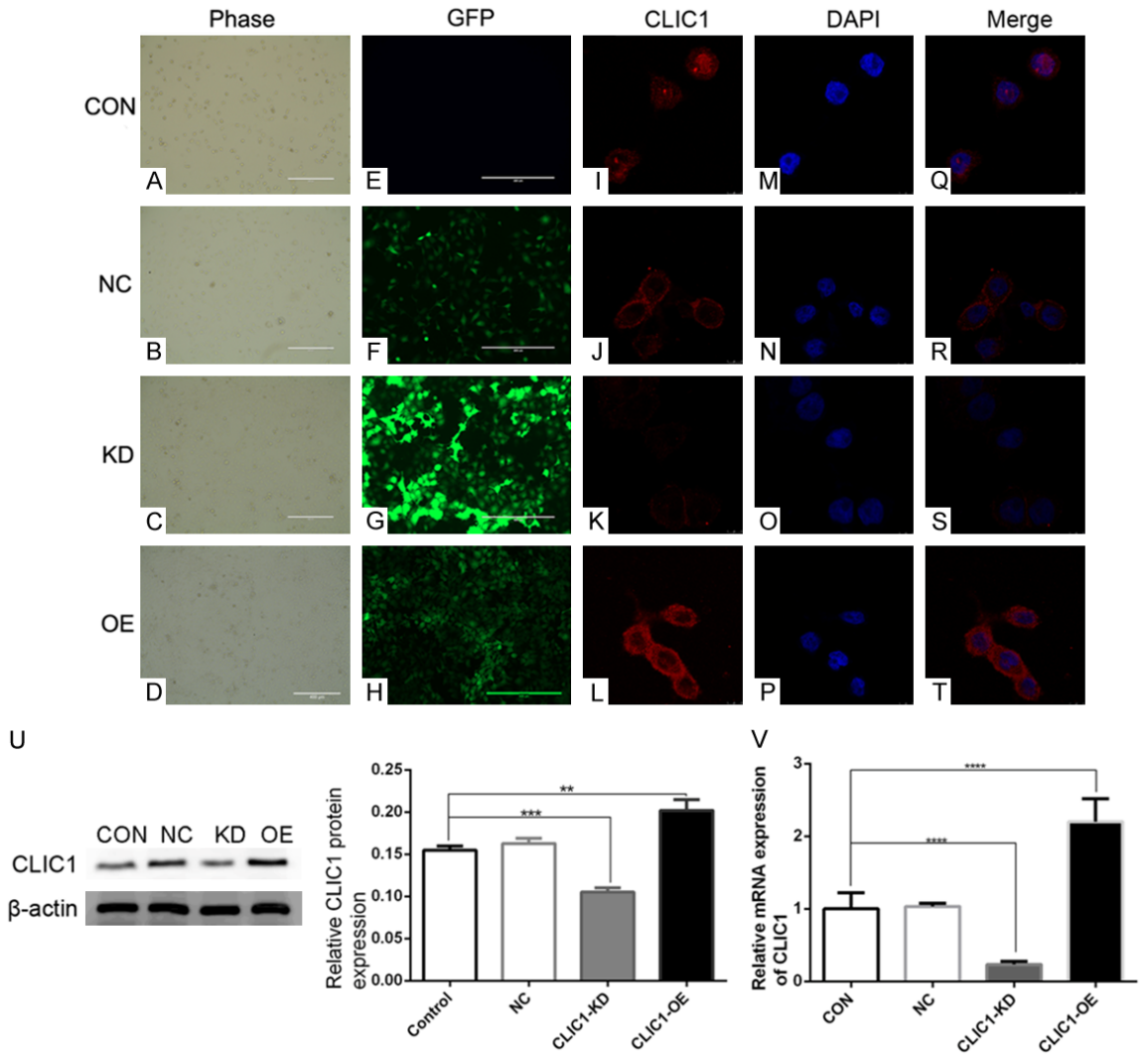

Figure 1. The untransfected SCC-15 cells and SCC-15 cells transfected with Lv-CLIC1, Lv-CLIC1-RNAi or shNC plasmids were analyzed by inverted fluorescence microscopy, IFA, western blotting and RT-PCR. A-H. Images of green fluorescent protein (GFP) expression obtained with an inverted fluorescence microscope. Cells in green indicate successful transfection. Scale bar: 400 μm. I-T. Immunofluorescence assays showed the subcellular location of CLIC1 expression. Red: anti-CLIC1 antibody; blue: nucleus. Scale bar: 10 μm. U, V. The relative expression of CLIC1 protein and mRNA in cell groups. Data are represented as the mean ± SD of three replicates. **P<0.01; ***P<0.001; ****P<0.0001. CON: control; NC: negative control; CLIC1-KD (KD): CLIC1 knockdown; CLIC1-OE (OE): CLIC1-overexpressing.

that of the control, shNC and CLIC1-overexpressing groups (P<0.001), and there was no significant difference among the control, shNC and CLIC1-overexpressing groups (P>0.05) (**Figure 2C**). Furthermore, we analyzed the expression levels of apoptosis-associated proteins and mRNA (caspase-3 and caspase-9) by western blotting and RT-PCR, and the results indicated that silencing CLIC1 significantly increased the caspase-3 and caspase-9 expression, compared with that of other groups (P<0.05) (**Figure 2D**, **2E**). These results showed that knockdown of CLIC1 inhibited OSCC cell viability and proliferation but promoted apoptosis in vitro, and CLIC1 upregulation promoted cell viability and proliferation.

CLIC1 promotes the migration and invasion of OSCC cells in vitro

Wound healing assays, transwell migration and invasion assays were performed to explore the effects of CLIC1 on OSCC cell migration and invasion in vitro. Wound healing assays showed

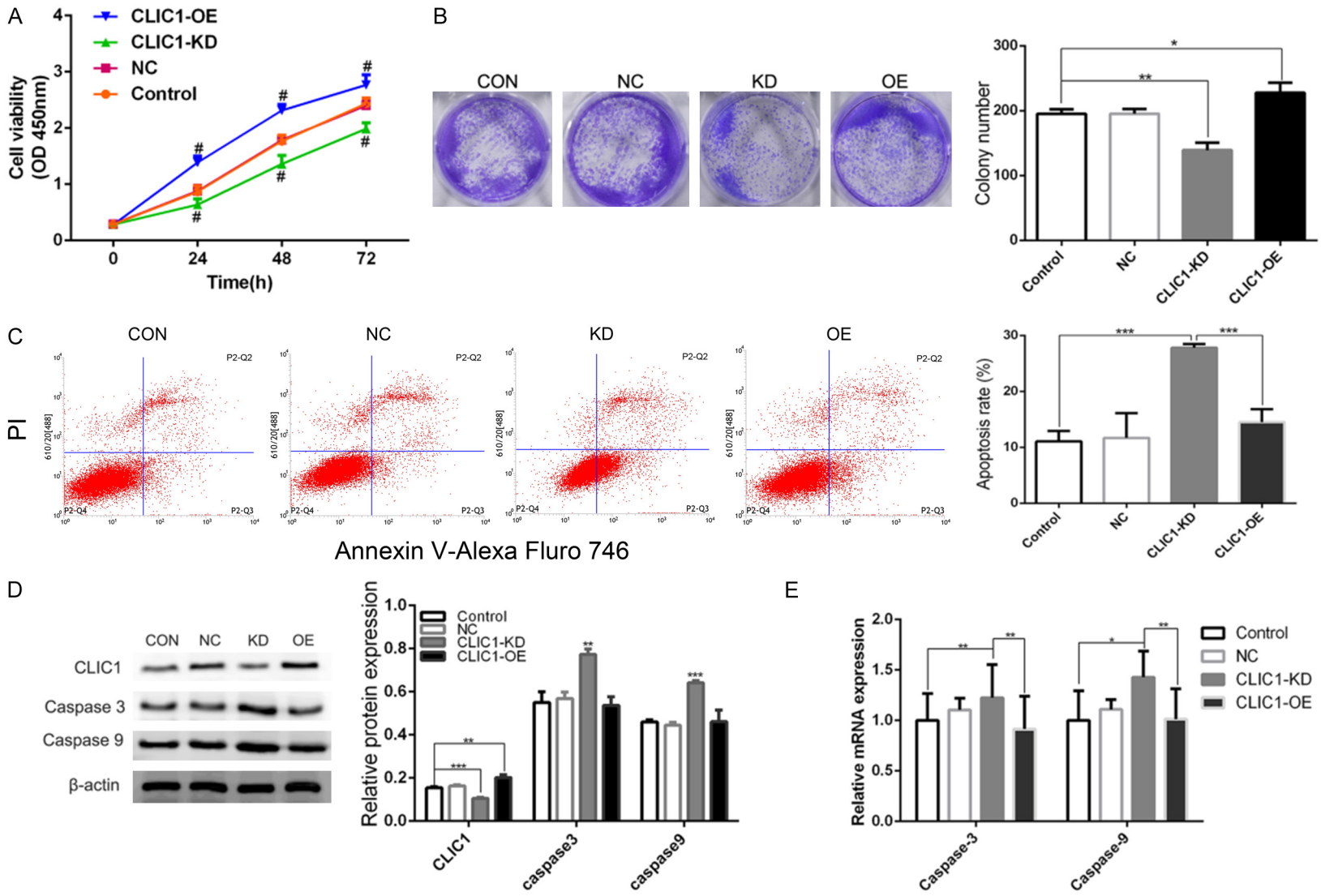

Figure 2. Effect of CLIC1 on viability and apoptosis of untransfected SCC-15 cells and SCC-15 cells transfected with Lv-CLIC1, Lv-CLIC1-RNAi or shNC plasmids in vitro. A. CCK-8 assays were performed to determine cell viability at 6, 24, 48, and 72 h after transfection. B. Representative images of cell colony formation and the number of colonies were counted. C. Flow cytometric analysis of OSCC cell apoptosis. The apoptosis rate was calculated by Q_2+Q_3. D, E. The relative expression of apoptosis related protein and mRNA (Caspase-3 and Caspase-9) in OSCC cell groups. Data are represented as the mean ± SD of three replicates. *P<0.05; **P<0.01; ***P<0.001; #P<0.0001.

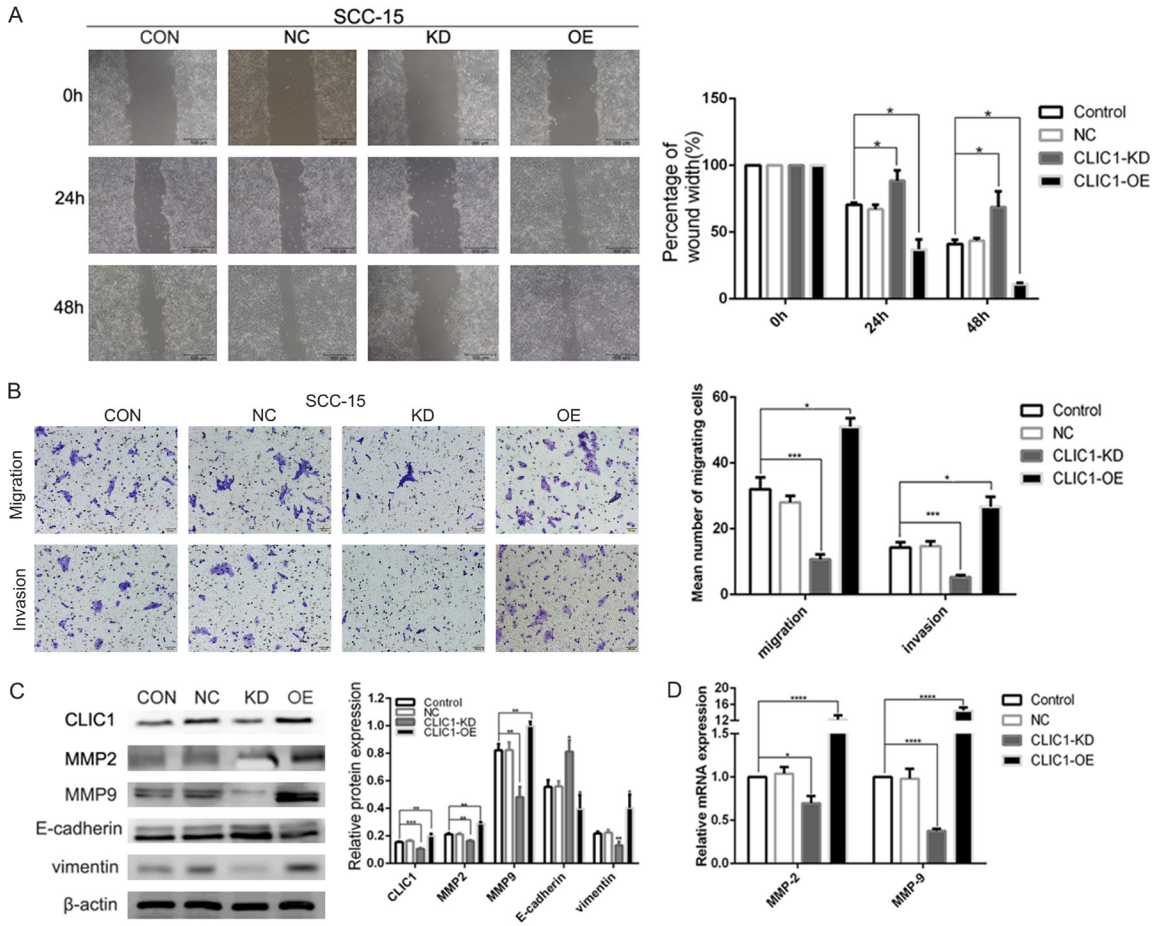

Figure 3. Effect of CLIC1 on the migratory and invasive abilities of OSCC cells in vitro. SCC-15 cells were untransfected (CON) or transfected with Lv-CLIC1 (OE), Lv-CLIC1-RNAi (KD) or shNC plasmids (NC). A. The motility of SCC-15 cell lines was determined by wound healing assays. The migration rates were calculated at o h, 24 h, and 48 h after transfection. Scale bar: 500 μm. B. Transwell migration and invasion assays showed the number of cells crossing the membrane. Scale bar: 50 μm. C and D. Western blotting and RT-PCR were performed to detect the expression of MMP-2, MMP-9, E-cadherin, and vimentin. Data are represented as the mean ± SD of three replicates. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

that the migration rate of CLIC1 knockdown cells was significantly slower than that of the control, shNC and CLIC1-ovexpressing groups (P<0.05). However, CLIC1 upregulation markedly increased the migration rate compared with that of the other three groups (P<0.05) (**Figure 3A**). Transwell assays showed similar results. CLIC1 silencing decreased the migrated and invaded OSCC cells that crossed the membrane (P<0.001), but upregulation of CLIC1 promoted these cells (P<0.05) (**Figure 3B**). MMP-2, MMP9, E-cadherin, and vimentin are the key factors in the process of epithelial-mesenchymal transition (EMT). We detected its expression by western blotting and RT-PCR. The results demonstrated that CLIC1 knockdown clearly suppressed the expression levels of MMP-2, MMP-9 and vimentin but increased the E-cadherin expression (P<0.05), while overexpression of CLIC1 promoted MMP-2, MMP-9 and vimentin expression and reduced the E-cadherin level (P<0.01) (**Figure 3C**, **3D**). These data indicated that CLIC1 promoted the migration and invasion of OSCC cells by regulating EMT in vitro.

CLIC1 promotes OSCC cell-mediated angiogenesis in vitro

Tube formation assays were used to study the effect of CLIC1 on angiogenesis of oral cancer cells. As shown in **Figure 4A**, tube formation was significantly suppressed in the HUVECs

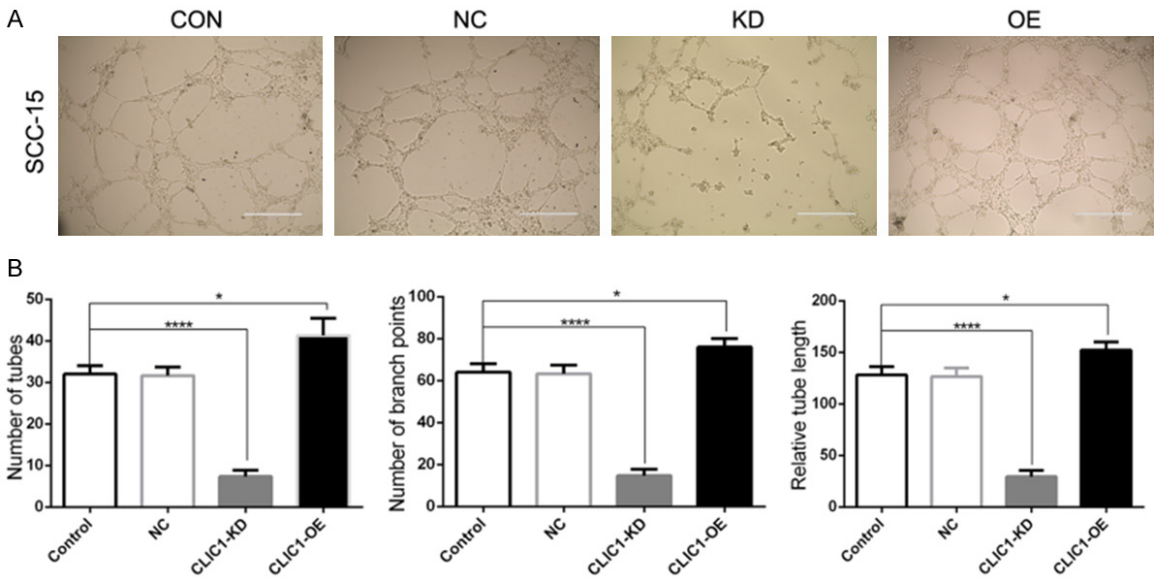

Figure 4. CLIC1 silencing inhibited the proangiogenic ability of OSCC cells in vitro. SCC-15 cells were untransfected (Control) or transfected with Lv-CLIC1 (OE), Lv-CLIC1-RNAi (KD) or shNC plasmids (NC), and the supernatant was applied to cultured HUVECs. A. Tube formation assays were performed to evaluate the effect of CLIC1 on the proangiogenic ability of OSCC cells. Scale bar: 400 μm. B. The number of tubules, number of branch points and relative tube length were calculated. Data are represented as the mean ± SD of three replicates. *P<0.05; ****P<0.0001 compared with the control and NC groups.

after the addition of CLIC1 knockdown OSCC cell supernatant compared with that of the control, shNC and CLIC1-overexpressing groups. The tube length, number of tubules and branch points were also significantly decreased (P<0.0001) (**Figure 4B**). Moreover, the upregulation of CLIC1 promoted tube formation, and the related statistical indicators were also significantly increased (P<0.05) (**Figure 4A**, **4B**). Thus, these results demonstrated that CLIC1 promotes oral cancer cell-mediated angiogenesis in vitro.

CLIC1 depletion improves OSCC cell sensitivity to cisplatin in vitro

The drug chemosensitivity of OSCC cells were measured by CCK-8 cytotoxicity assays. The inhibition rate of CLIC1 knockdown cells was greater than that of control, shNC and CLIC1-overexpressing groups along with the increase in cisplatin concentration (P<0.05) (**Figure 5C**). CLIC1 silencing reduced the mean half maximal inhibitory concentration (IC_{50}) value of cisplatin in OSCC cells compared with control and shNC cells (P<0.05), and there was no significant difference between among the control, NC and overexpression groups (P>0.05) (**Table 2**). Furthermore, the apoptosis of transfected OSCC cells cultured with CDDP was analyzed using FCM. The apoptosis rate of CLIC1 knockdown cells was markedly increased compared with that of the untransfected, shNC and CLIC1-ovexpressing cells (P<0.01) (**Figure 5A** and **5B**). These data indicated that CLIC1 depletion improves the OSCC cell drug sensitivity to cisplatin in vitro.

CLIC1 depletion suppress the growth of OSCC in vivo

Untransfected cells and cells transfected with Lv-CLIC1, Lv-CLIC1-RNAi or shNC were subcutaneously injected into the dorsal area of the mice to generate the tumor growth models. There was a notable reduction in tumor volume, size and weight of the CLIC1 knockdown group compared with control, shNC and CLIC1-ovexpressing groups (P<0.05), while those of the CLIC1-overexpressing group were larger (P<0.05) (**Figure 6A-C**). The sample tissues were verified for OSCC by HE staining, and there were no obvious lesions in the liver and kidneys (**Figure 6D**). IHC assays showed that the CLIC1, MMP-2, MMP-9, MMP-13, P-p38 and P-ERK protein levels in the CLIC1 knockdown group were significantly lower than those of the other groups, while overexpression of

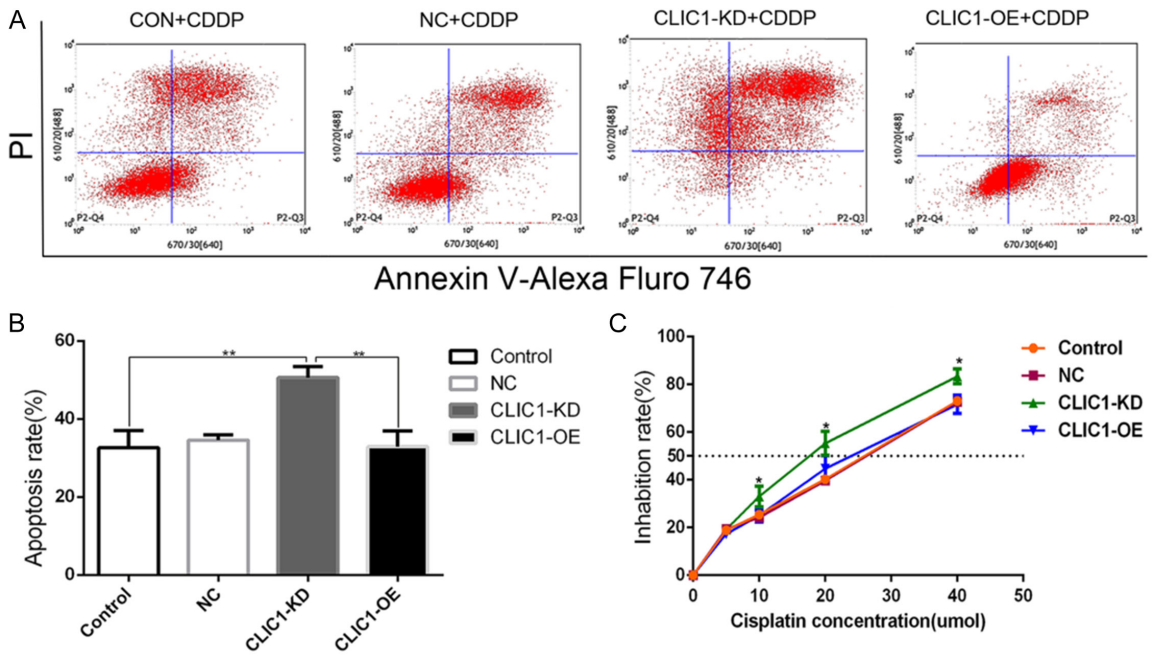

Figure 5. CLIC1 knockdown increased the cisplatin chemosensitivity of OSCC cells and its apoptosis rate. SCC-15 cells were untransfected (Control) or transfected with Lv-CLIC1 (OE), Lv-CLIC1-RNAi (KD) or shNC plasmids (NC) and then cultured with cisplatin. A and B. The apoptosis rates of SCC-15 cells cultured with 20 μmol cisplatin for 24 h after transfection were detected by FCM. C. CCK-8 cytotoxicity assays showed the drug sensitivity of cisplatin for OSCC cells. Data are represented as the mean ± SD of three replicates. *P<0.05 compared with CON, NC or OE group; **P<0.01.

Table 2. The IC_{50} values of CDDP in SCC-15 cell lines after transfection

Drug	Group	IC_{50} (umol)
CDDP	Control	22.20±3.01
CDDP	NC	22.68±3.29
CDDP	CLIC1-KD	15.71±1.59*
CDDP	CLIC1-OE	21.63±3.42

*P<0.05 compared with the CON, NC or CLIC1-OE groups. CON: control; NC: negative control; CLIC1-KD: CLIC1 knockdown; CLIC1-OE: CLIC1-overexpressing.

CLIC1 promoted the CLIC1, MMP-2, MMP-9, MMP-13, and p-ERK expression (**Figure 6E**, **6F**). The western blotting showed that CLIC1 silencing decreased the p-ERK protein level and increased the p-p38 expression, compared with those of the control and shNC groups (**Figure 6G**). Thus, these data suggested that CLIC1 promotes the growth of oral cancer and CLIC1 is involved in MAPK signaling pathways in vivo.

CLIC1 affects the protein expression of integrin family proteins and MAPK signaling pathways

Integrin (ITG) family proteins were shown to be involved in the development of oral cancer [19-21], and a previous study reported that CLIC1 silencing regulated the expression of ITG proteins in vitro [13]. We input the CLIC1, ITGαv, ITGβ1, and MAPK1 (ERK, p38) genes into the GeneMANIA program (http://genemania.org/) to predict the signaling pathways. The results showed that CLIC1, ITGαv, ITGβ1, MAPK1 were in the same signal network, which indicated that there might be a regulatory relationship among them (**Figure 7**). Then, western blotting assay was performed to analyze the expression levels of CLIC1, ITGαv, ITGβ1, p-ERK, p-p38 and several downstream proteins of the MAPK signaling pathway. CLIC1 silencing notably decreased the OSCC cell protein levels of ITGαv, ITGβ1, p-ERK, vimentin, MMP2 and MMP9 but increased the p-p38, caspase3, caspase9, and E-cadherin protein expression, while CLIC1 overexpression promoted the ITGαv, ITGβ1, p-ERK, vimentin, MMP2 and MMP9 levels and decreased the E-cadherin level (P<0.05). The expression levels of ERK, p38 remained unchanged (**Figure 8A-C**). Thus, these results demonstrated that CLIC1 affects the protein expression of ITG family proteins and the MAPK/ERK and MAPK/p38 signaling pathways.

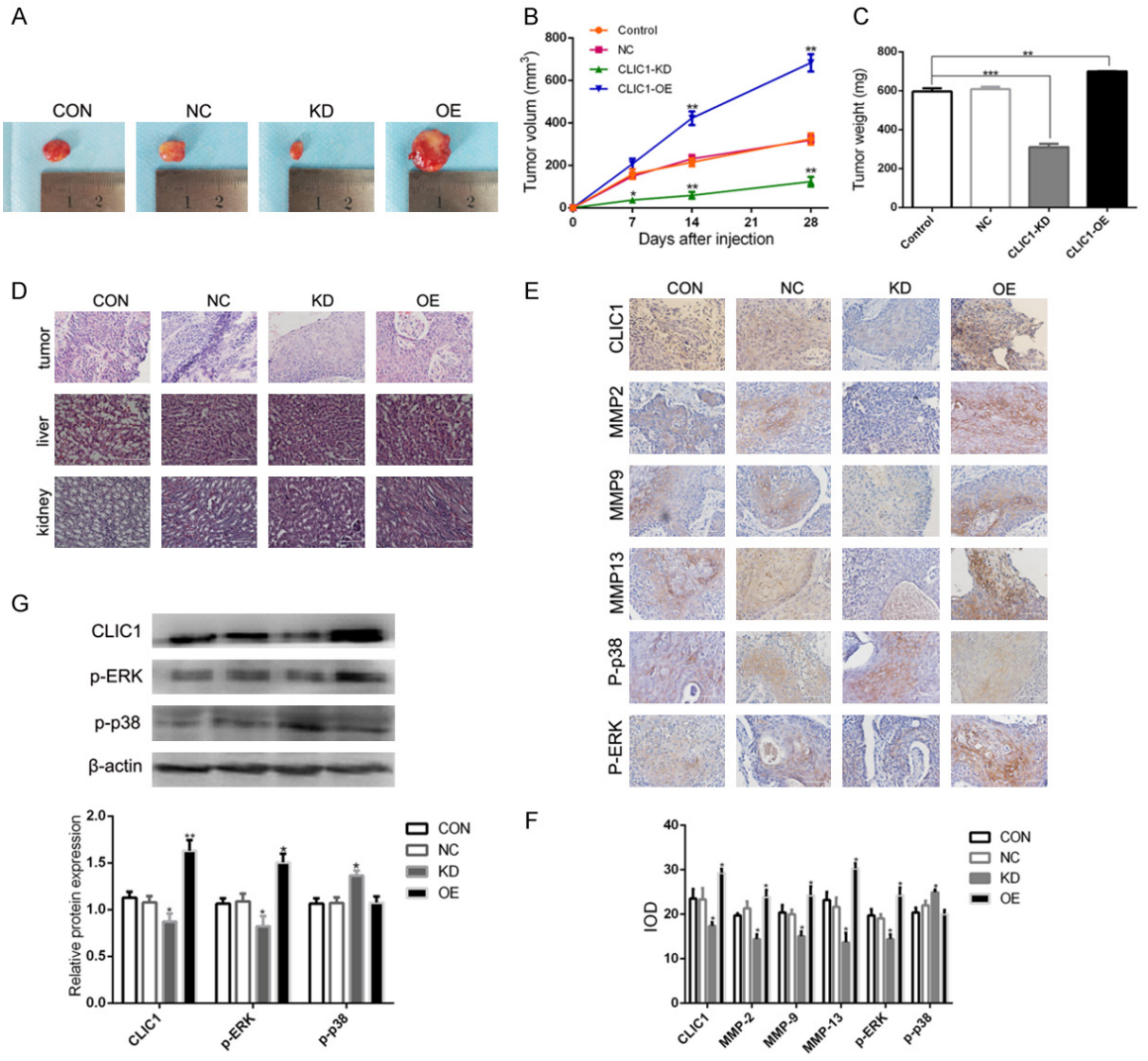

Figure 6. CLIC1 depletion hindered tumorigenesis and was involved in the MAPK signaling pathway. Female 5-week-old BALB/C nude mice were subcutaneously injected with untransfected OSCC cells or OSCC cells transfected with Lv-CLIC1, Lv-CLIC1-RNAi or shNC plasmids. A. Representative tumor photographs from each group. B. The tumor volume was measured once a week for 4 weeks after injection. C. Tumor weights were measured after dissection. D. HE staining of tumors, liver and kidney from xenografts. Scale bar: 100 μm. E, F. Immunohistochemical analysis was performed to determine the expression of CLIC1, MMP2, MMP9, MMP13, p-ERK, and p-p38 in tumor samples from xenografts. Scale bar: 100 μm. G. Western blotting assay confirmed the protein expression of p-ERK and p-p38 in xenograft tumor tissues. tuactin was used as a loading control. Data are represented as the mean ± SD of three replicates. *P<0.05; **P<0.01; ***P<0.001.

Discussion

CLIC1 is an active ionic channel or signal transducer that is involved in many physiological and pathological processes [4, 22]. Previous studies have demonstrated that CLIC1 plays an important role in the progression of several malignant tumors [13-18]. Karsani SA compared the whole cell proteome of three oral cancer cell lines with three normal primary cells by two-dimensional gel electrophoresis (2DE) and IPA analysis and predicted that CLIC1 may be a potential biomarker for oral cancer [23], but no in vitro and in vivo analyses have confirmed this theory. The early results of our group showed that the expression of CLIC1 in OSCC tissues and the blood of cancer patients increased significantly, and the upregulation of CLIC1 was distinctly associated with the clinical and pathological stages, tumor size, and overall survival rate (**Figure 9**) [18]. As a series of study, this research aimed to explore the

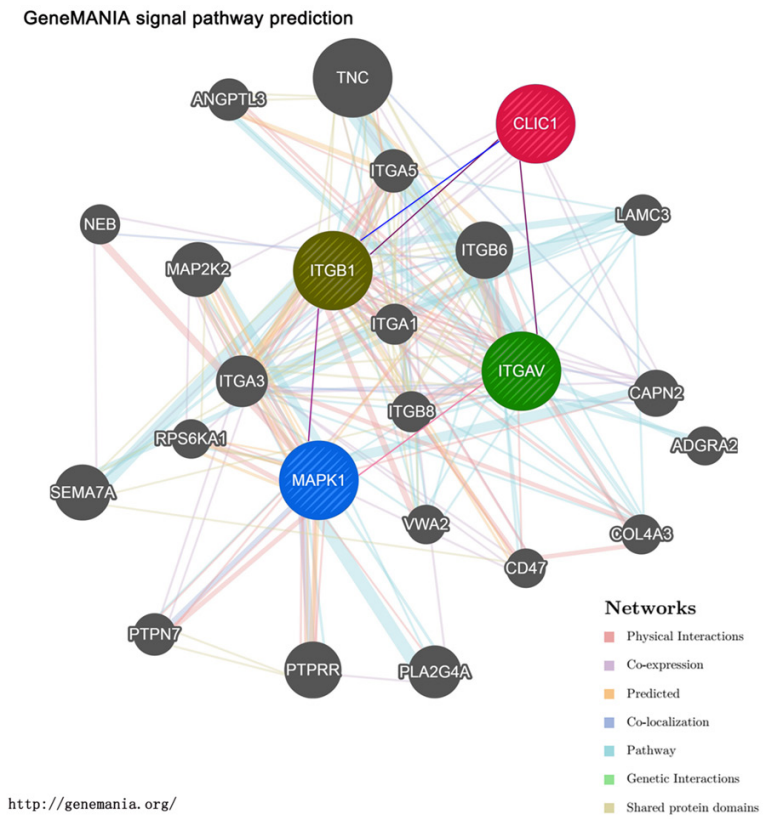

Figure 7. CLIC1 regulated the relationship of integrin genes and MAPK family genes by GeneMANIA in signal pathway prediction.

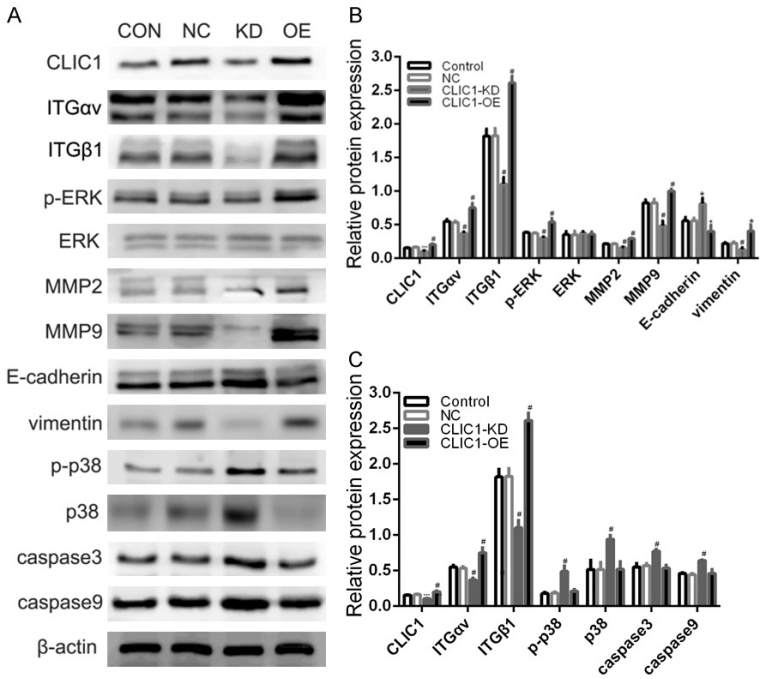

Figure 8. CLIC1 activated the ITGs/MAPK signaling pathway. The related proteins in the ITGs/MAPK signaling pathway are detected by Western blot, including ITGαv, ITGβ1, ERK1/2, p-ERK1/2, p38, p-p38, MMP2, MMP9, E-cadherin, vimentin, caspase3, and caspase9 in each CLIC1 transfected OSCC cell group. β-actin was used as a loading control. #P<0.01; ***P<0.001.

relationship between CLIC1 and OSCC at the cellular level and the possible related molecular mechanisms.

The changes in the gene structure and function of tumor cells results in the following characteristics of tumor cells: insensitivity to growth inhibition signals, evasion of apoptosis and unlimited proliferation potential [24]. Recently, CLIC1 was reported to regulate the proliferation and apoptosis of tumor cells. Kobayashi found that CLIC1 depletion inhibited cell proliferation and induced apoptosis in esophageal squamous cell carcinoma (ESCC) [14]. Similar results have been found in gastric cancer cells [13]. In hepatocellular carcinoma research, CLIC1 overexpression increased cell viability [12]. In the present study, we found that CLIC1 silencing inhibited the OSCC cell viability and proliferation but promoted apoptosis, and the upregulation of CLIC1 was positively correlated with cell viability and proliferation. The results of tumor growth in vivo also confirmed this conclusion. As key factors in apoptosis, caspase-3 interacts with caspase-9 to induce apoptosis and its sequential activation plays an important role in the execution phase of cell apoptosis [25]. We measured the RNA and protein levels in OSCC cells and found that silencing CLIC1 increased the expression of caspase-3 and caspase-9. Thus, we inferred that CLIC1 promotes the viability and proliferation of OSCC cells and is involved in OSCC cell apoptosis.

Local infiltration and distant metastasis are the most important biological characteris-

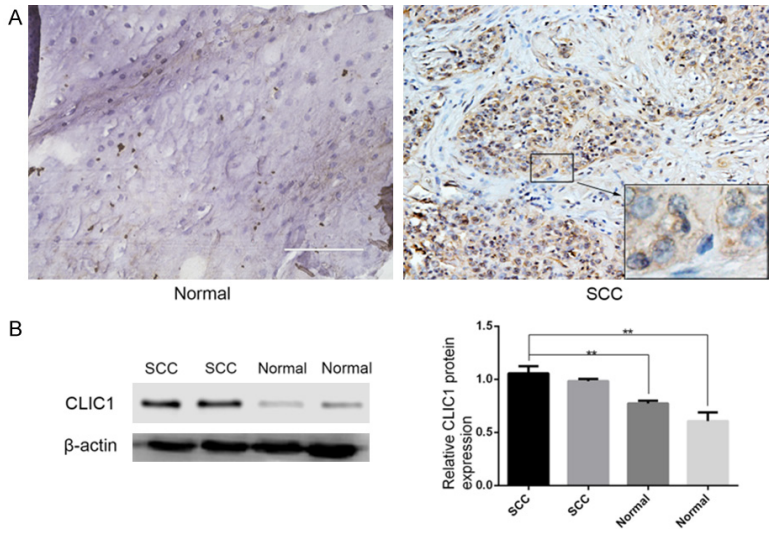

Figure 9. Expression of the CLIC1 protein in cancerous and adjacent noncancerous tissues of OSCC patients. A. Immunohistochemistry assay of CLIC1 in cancerous and adjacent noncancerous tissues from OSCC patients. Yellow-brown coloration indicates CLIC1 protein expression. Scale bar: 100 μm. B. Western blot analysis of CLIC1 in OSCC tissues and noncancerous tissue. Data are represented as the mean ± SD of three replicates. **P<0.01.

tics of malignancies [26]. CLIC1 has diametrically opposite roles in tumor spreading and metastasis. Wang JW showed that CLIC1 overexpression markedly promoted the motility and invasion of gallbladder cancer cells, while CLIC1 knockdown significantly decreased it [27]. Similar results have been found in colon cancer [28], gastric cancer [13], and breast cancer [29]. However, a study focused on ESCC stated that CLIC1 depletion promoted cell migration and invasion [14]. Thus, we hypothesized that CLIC1 is involved in tumor spreading and metastasis. In our study, the results were the same as those in gallbladder cancer. Moreover, we detected the expression levels of MMP2, MMP9, vimentin and E-cadherin. EMT is an important biological process for the migration and invasion of malignant tumor cells derived from epithelial cells. EMT is primarily characterized by the reduction in expression of cell adhesion molecules (such as E-cadherin) and the transformation of the cell keratin cytoskeleton into a waveform protein (Vimentin) [30]. MMPs degrade various protein components in the extracellular matrix (ECM), including the histological barrier against tumor cell invasion, MMP2 and MMP9 are also the vital factors in the process of EMT. Our results showed that CLIC1 depletion inhibited the expression of MMP2, MMP9, and vimentin but promoted E-cadherin expression. In addition, completely opposite results were observed in the CLIC1-overexpressing cells. These results suggested that CLIC1 promotes the migration and invasion of OSCC cells.

Microvascular growth induced by tumor cells is an important factor for tumorigenesis, growth, invasion and metastasis. VEGF is a key regulator of tumor angiogenesis [31]. Research on hepatocellular carcinoma has shown that that CLIC1 knockdown reduced the expression of VEGF, while overexpression of CLIC1 decreased its expression [12]. However, previous studies did not investigate the effect of CLIC1 on the tube formation of HUVECs. Our results intuitively demonstrated that knockdown of CLIC1 in OSCC cells inhibited the angiogenesis of HUVECs; in contrast, overexpression of CLIC1 promoted it.

Chemotherapy is one of the most effective treatments for cancer, but the curative effect is affected by drug resistance. Chen Y found that CLIC1 knockdown increased the chemosensitivity to cisplatin in ovarian cancer cells [32]. In contrast, a study in gallbladder cancer showed that dysfunction or downregulation of CLIC1 could partially decrease the anti-neoplastic effects of metformin, while upregulated CLIC1 increased drug susceptibility [16]. Our results demonstrated that the drug susceptibility of OSCC cells to cisplatin was increased after CLIC1 knockdown, and the apoptosis rate was also increased. Cisplatin is the first developed platinum-based drug, and the generally accepted antitumor mechanism is that platinum drugs act on DNA, form adducts with DNA, limit DNA unwinding and inhibit DNA replication [33]. The antitumor mechanism of metformin involves AMPK pathway activation, cell cycle arrest, autophagy, and tumor stem cell killing [34]. Differences in anti-tumor mechanisms might lead to opposite trends in drug sensitivity in CLIC1 knockdown OSCC cells. In a word, these results

demonstrated that CLIC1 depletion increased the chemosensitivity to cisplatin in OSCC cells and led to incrased apoptosis.

The present studies indicated that CLIC1 was involved in cancer progression, but the specific mechanism remained unclear. Wang P found that CLIC1 regulates colon cancer cell migration and invasion via ROS-mediated MAPK/ERK signaling pathway [28]. A study on gastric cancer demonstrated that CLIC1 may regulate the expression of ITG family proteins and lead to sequential activation of the PI3K/AKT, MAPK/ERK and MAPK/p38 pathways [13]. ITGs are transmembrane receptors that facilitate cell ECM adhesion and mediate bidirectional signal transduction from the ECM to the cell [35]. Several studies have reported that ITG family proteins play an important role in the apoptosis, invasion and migration of OSCC, and ITGαv regulated the proliferation and invasion of OSCC cells via MAPK/ERK signaling pathway by interacting with ITGβ [19-21, 36]. The ITGs also may affect the MAPK/ERK and MAPK/p38 pathways in other tumors [37, 38]. In addition, iTRAQ technology showed that CLIC1 depletion decreased the expression of ITGs [13]. Thus, we hypothesized that CLIC1 interacts with ITG proteins, resulting in activation of the MAPK signaling pathway to regulate OSCC progression. The signal pathway prediction from GeneMANIA was consistent with our hypothesis; the CLIC1, ITGαT, ITGβ1, and MAPK1 genes were in the same signal network and had connections with each other (**Figure 7**). Phosphorylated ERK plays an important role in the activation of the MAPK/ERK signaling pathway, and it is involved in the regulation of proliferation, differentiation and invasion of tumor cells [39]. Western blotting results showed that CLIC1 silencing decreased the ITGαv, ITGβ1, p-ERK, MMP2, MMP9, and vimentin protein levels in vitro but increased the E-cadherin expression. The migration and invasion of OSCC cells were also suppressed, while upregulation of CLIC1 resulted in the completely opposite effect on protein expression and biological behaviors. P38-phosphorylation is critical for the activation of the MAPK/p38 pathway and is mainly associated with inflammation, stress and apoptosis in oral cancer [40]. In the present study, the expression levels of p-p38, caspase-3 and caspase-9 were increased when CLIC1 was depleted, and at the same time, the apoptosis rate also increased. The results from xenograft tumor tissues also showed that CLIC1 knockdown decreased the p-ERK protein level but promoted p-p38 expression. MMP2, MMP9, vimentin and E-cadherin are the key factors of ECM in tumor migration and invasion. Caspase-3 and caspase-9 also play an important role in apoptosis. Thus, these results indicated that CLIC1 might regulate and interact with ITGs, and then, activate the MAPK/ERK and MAPK/p38 pathways and is thus involved in OSCC cell invasion and apoptosis. In summary, our study demonstrated that CLIC1 knockdown significantly suppressed the proliferation, vitality, migration, invasion and angiogenesis of OSCC cells but promoted apoptosis and increased the chemosensitivity to cisplatin. In contrast, CLIC1 upregulation showed the completely opposite effects. In addition, changes in CLIC1 altered the expression of ITGαv and ITGβ1, which regulate the signaling pathway cascades. Thus, these findings indicated that CLIC1 may play a critical role on the progression of OSCC cells in vitro, and we speculated that its potential mechanism may be related to the regulation of ITGαv and ITGβ1, which lead to the activation of the MAPK/ERK and MAPK/p38 pathways.

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Disclosure of conflict of interest

None.

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