



Chloride intracellular channel 1 promotes esophageal squamous cell carcinoma proliferation via mTOR signalling

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ABSTRACT

Objectives: To investigate the clinical significance of Chloride Intracellular Channel 1 (CLIC1) expression in esophageal squamous cell carcinoma (ESCC) and its functional contribution and molecular mechanisms to the progression of ESCC.

Methods: CLIC1 expression was analyzed by immunohistochemistry (IHC) in a cohort of 86 ESCC tissue specimens and paired normal adjacent esophageal tissues. Associations between clinicopathological features of ESCC and CLIC1 expression were determined. *In vitro* analyses examined CLIC1 expression in the ESCC cell lines KYSE150 and TE1 using RT-PCR and Western blotting. The downstream pathways of CLIC1 were detected by lentiviral shRNA knockdown and subsequent proteomic analyses. CLIC1 siRNA knockdown was performed in ESCC cell lines KYSE150 and TE1 and the functional effects of CLIC1 on the growth and proliferation of ESCC cells were evaluated combined with cell viability and colony formation assays; the mTOR signaling pathway-related proteins were detected by Western blotting based on the previous proteomic data.

Results: CLIC1 expression was significantly increased *in ex vivo* ESCC tissues compared with corresponding normal tissues, and the up-regulation was associated with clinical tumor node metastasis (TNM) classifications. Knockdown of CLIC1 inhibited *in vitro* cell proliferation of ESCC cell lines KYSE150 and TE1. CLIC1 knockdown down-regulated the protein expression of p-mTOR and the downstream targets Rictor and p-4EBP1 in both KYSE150 and TE1 cell lines. And the CLIC1 knockdown induced inhibition of cell proliferation on ESCC cells could be rescued by mTOR overexpression.

Conclusions: CLIC1 expression increases during esophageal carcinogenesis and it may functionally contribute to the progression of ESCC through growth promotion effects by promoting the mTOR and downstream signaling pathway. CLIC1 therefore constitutes a candidate molecular biomarker of ESCC.

Introduction

Chloride intracellular channel proteins (CLIC) belong to the glutathione S-transferase (GST) fold superfamily and exist in both soluble and transmembrane forms [1,2]. There are seven high homologous members of the CLIC family (CLIC1-6, CLIC5B) in vertebrates with the archetypal CLIC1 gene being originally cloned from activated human macrophages

[3]. CLIC1 consists of a single-pass membrane surface protein of 241 amino acids that plays essential roles in various physiological functions, including regulation of cell volume, organelle acidification and modulation of ion homeostasis [4–6]. For example, depletion of CLIC1 impairs phagosome acidification in dendritic cells, thus inhibiting T cells activation by modulating antigen presentation by dendritic cells [7]. CLIC1 is also a sensor and effector in oxidative stress by regulating ROS

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production and inflammatory cytokine release in endothelial cells [8]. The blockade of CLIC1 in microglia cells also impairs the production of ROS and nitrites [9]. Moreover, the targeted depletion of CLIC1 using the chloride channel inhibitor IAA-94 has been reported to inhibit the release of IL-1 β in macrophages by regulating the activation of NLRP3 inflammasome [10,11].

In the cancer context, CLIC1 expression appears ubiquitous in many human cancer tissues where it has been reported to act as an oncogene, including gastric cancer, colorectal cancer and hepatocellular carcinoma [12]. CLIC1 up-regulation in some malignant tumors has also been associated with poor prognosis, suggesting it is a potential prognostic marker and potentially a target for anti-tumor therapy [13,14]. At the functional level, CLIC1 appears to contribute to the proliferation, migration and invasion of some cancer cells [15,16]. Different studies have elucidated the possible involvement of CLIC1 in the formation and progression of cancers, for example, it promotes cell proliferation of gastric cancer by regulating MAPK/AKT pathway [17], and in hepatocellular carcinoma (HCC) it promotes tumorigenesis through a positive feedback loop with MYC and predicts worse outcomes for HCC patients [14]. CLIC1 can also promote tumor metastasis by recruiting PIP5K1A and PIP5K1C from the cytoplasm to the leading edge of the plasma membrane [18]. However, the role of CLIC1 in human esophageal cancer (EC), particularly esophageal squamous cell carcinoma (ESCC) which represents over 90% of EC patients in China remains to be elucidated [19].

The PI3K/Akt signaling pathway is fundamentally involved in regulating cellular activities, including survival, proliferation, transcription, translation and metabolism [20,21]. Moreover, the PI3K/Akt signaling pathway is abnormally activated in most human cancers, including carcinomas of the breast, ovary, colon and rectum, and liver [22–25]. Notably, in a mouse model of melanoma, inhibition of the PI3K/Akt signaling pathway reduced brain metastases [26] while the upregulation of PI3K/Akt signaling promotes the proliferation of renal cell carcinoma cells [27]. Furthermore, the PI3K/AKT signaling pathway was found to promote epithelial-mesenchymal transition (EMT) in ovarian cancer cells by upregulating the transcription factors Snail and Slug [28]. However, presently there are few related studies detailing whether the PI3K/Akt signaling pathway promotes the progression of ESCC and consequently further exploration is required. mTOR is a serine/threonine protein kinase in the PI3K related kinase family, and is one of the downstream molecules of the PI3K/Akt signaling pathway [29]. mTOR can form two protein complexes, namely mTOR complex 1 (mTORC1) including mTOR, Raptor and mLST8, and mTOR complex 2 (mTORC2) including mTOR, Rictor and mLST8 [30–32]. mTORC1 can participate in the metabolism of intracellular proteins, lipids, nucleotides and glucose to promote cell growth and proliferation, while mTORC2 can regulate cell survival and cell migration [33–36]. Studies have shown that mTORC1 can phosphorylate 4EBP1 to promote the proliferation of breast cancer cells [37]. Furthermore, disrupting the assembly of mTORC2 inhibits the growth and survival of PTEN-deficient prostate cancer [38]. However, there are still few reports about the participation of mTOR in ESCC, which is worthy of further study.

Our study sought to define whether CLIC1 represents a potential biomarker for ESCC and whether its expression correlates with traditional clinicopathological features of ESCC. Based on this, we employed quantitative proteomics to identify potential downstream pathways and aimed to elucidate the molecular mechanism of oncogenic CLIC1 in ESCC cells.

Methods

ESCC specimens and ethics

All specimens were obtained from patients who had not received any chemoradiotherapy, immunotherapy or combinational therapy at the

First Affiliated Hospital of Anhui Medical University between 2015 and 2016. Primary tumor specimen and the corresponding non-cancerous tissue (located more than 5 cm away from tumor margins) were collected. All diagnoses were independently confirmed by two professional pathologists. The research was approved by the Human Ethics Review Committee of Anhui Medical University (20190402) with written informed consent obtained from each participating patient.

Cell culture

The human ESCC cell line ECA109 was obtained from the Culture Collection of the Chinese Academy of Sciences (Shanghai, China); KYSE30, KYSE70, KYSE150, KYSE520, TE1 and the normal esophageal epithelial cell line Het-1A were all obtained from Kebai Biological Technology (Nanjing, China). Cells were maintained in RPMI medium (Hyclone, USA) supplemented with 10% (v/v) fetal bovine serum (Hyclone, USA) in a humidified incubator at 37°C with 5% CO₂.

TMT and bioinformatic analyses

Suspensions of the indicated cells were sonicated three times on ice using a high intensity ultrasonic processor (Scientz) in lysis buffer (8 M urea, 1% Protease Inhibitor Cocktail). The remaining debris was removed by centrifugation at 12,000 g at 4°C for 20 min. Finally, supernatants were collected and protein concentrations were determined with the BCA Kit according to the manufacturer's instructions (Sangon Biotech, China). The samples were then trypsin digested before TMT Labeling-based proteomics and LC-MS/MS analysis was performed by Jingjie Biotech Co., Ltd. Hangzhou, China.

Mass spectrometric data were searched against the UniProt/SwissProt human proteome database using MaxQuant v.1.5.2.8 (<http://www.maxquant.org/>) to identify protein hits in each sample. Subcellular localization of hits was predicted using Wolfpsort v.0.2, an updated version of PSORT/ PSORT II (<http://www.genscript.com/wolf-psort.html>). Differentially expressed proteins (DEPs) between and among groups were analysed using the R vegan package (Version 2.0-2; Oksanen et al., 2011) in R v.2.8.1. Student's *t* and ANOVA tests were carried out to determine the statistical significance between two and among more groups, respectively (SPSS standard version 22.0, SPSS, Inc.). DEPs and heatmap were analysed using TnnDiagram package in R v.2.8.1, respectively. Identified proteins domain functional description were annotated by InterProScan (<http://www.ebi.ac.uk/interpro>) based on protein sequence alignment method. Encyclopedia of Genes and Genomes (KEGG) database was used to identify enriched pathways by a two-tailed Fisher's exact test to evaluate the enrichment of the DEPs against all identified proteins. Pathways with corrected *p*-values <0.05 were considered significant. These pathways were classified into hierarchical categories according to the KEGG website (<https://www.genome.jp/>).

Lentiviral gene transduction and siRNA transfection

The lentivirus-based inducible knockdown system targeting human CLIC1 along with the negative control were purchased from Genechem (Shanghai, China). Cells were infected with lentivirus at MOI of 40 at confluence of 20% with 5–8 μ g/ml of polybrene. The siRNAs targeting CLIC1 (siCLIC1-1, 5'-CUUCAUGUUACCACCGUU-3', siCLIC1-2, 5'-GUGGAUGAAACCAGUGCUG-3') were synthesized by GenePharma (Shanghai, China). Transfections were performed using the Lipofectamine™ RNAiMAX (Invitrogen, USA) according to the manufacturer's instructions.

Immunohistochemistry

Tissues were fixed in formalin and embedded in paraffin. Histological sections of 4 μ m were adhered to slides, deparaffinized, hydrated

and antigen retrieval performed in a microwave oven. After cooling to room temperature, sections were incubated in 10% (v/v) H₂O₂ solution for 10 min and then incubated with CLIC1 antibodies (1:250, SC-134859; Santa Cruz biotechnology, USA) or normal rabbit serum (1:250 dilution) as a negative control. Diaminobenzidine-based detection and counterstaining with hematoxylin was performed with an Immunohistochemistry kit (PV-6001, Zsbio, China) according to the manufacturer's instructions. CLIC1 expression was graded semi-quantitatively, combining the staining intensity with percentage of positive tumor cells. Immunoreactivity was blindly evaluated by two professional pathologists according to immunoreactivity score (IRS) system as previously described [29]. For CLIC1 analysis, we combined weak positive and negative staining cases as low expression, and moderate and strong positive staining as high expression.

Real-time PCR

Total RNA was extracted from frozen tissues using TRIzol solution (Ambion, USA) and quantitated using an UltrasecTM 2100 pro UV/Visible Spectrophotometer (Amersham Biosciences, Uppsala, Sweden). Reverse transcription reactions into cDNA were then performed using TaKaRa Reverse Transcription Reagents (TaKaRa Bio, Japan) according to the manufacturer's instructions. Primers were synthesized by Sangon Biotech (Shanghai, China) as follows: CLIC1 forward: 5'-AATCAAACC-CAGCACTCAATG-3', reverse: 5'-CAGCACTGGTTTCATCCACTT-3'. GAPDH forward: 5'-GAAGGTGAAGGTCGGAGTC-3' and reverse: 5'-GAAGATGGTATGGGATTTC-3'. Real-time PCR was performed using NovoStart SYBR qPCR Supermix Plus reagents (Novoprotein, China) on the QuantStudio6 Flex instrument (ABI, USA) using 10 µl reactions. Cycling conditions were: denaturation at 95°C for 20 s, annealing at 58°C for 20 s, and extension at 72°C for 30 s with 35 cycles. Relative gene expression was analyzed by the 2^{-ΔΔCt} method.

Western blotting

Total cell lysates were separated by SDS-PAGE and transferred to PVDF membranes (Millipore, USA). Membranes were blocked by 5% (m/v) skim milk in TBST and after washing in TBST for three times, the membrane was incubated with primary antibodies against CLIC1 (14545-1-AP, Proteintech, China), p-Akt (#4060, CST, USA), Akt (#2920, CST, USA), p-mTOR (2971S, CST, USA), mTOR(2972S, CST, USA), Rictor (2114T, CST, USA), p-S6K (9206S, CST, USA), S6K (2708T, CST, USA), p-S6 (5364S, CST, USA), S6 (2217S, CST, USA), p-4EBP1 (ab173371, Abcam, UK), 4EBP1 (60246-1-Ig, Proteintech, China) or β-actin (66009-1-Ig, Proteintech, China) overnight at 4°C. After washing, membranes were incubated with appropriate HRP-conjugated secondary antibodies for 1 h at room temperature before detection using ECL (34094, Pierce, USA). Densitometric analyses were conducted with Image J (ImageJ, USA) and relative CLIC1 expression normalized against β-actin.

Cell proliferation assay

Cell proliferation was analyzed by the CCK-8 kit (Dojindo, Japan) following the manufacturer's instructions. Briefly, relative cell numbers were assessed by measuring the optical density of cell culture medium at 450 nm in a microplate reader (Molecular Devices, USA). Assays were performed in triplicate.

Colony formation assay

Two thousand cells were seeded into 6 well plate and cultured for 10-12 days, changing the culture medium every three days. Cells were washed three times with PBS, then fixed with precooled (-20°C) absolute methanol for 15 min and stained with crystal violet solution for 15 min at room temperature. After drying the plates, the colonies in each well

were counted.

Statistical analyses

Data are presented as the means ± SEM of at least three independent experiments. Prism 5 software (GraphPad, USA) was used for statistical analyses where the Student's *t* test was used to assess differences between different groups. *P* < 0.05 was considered statistically significant.

Results

CLIC1 is upregulated in ESCC tissues and is associated with advanced tumor stage

As expounded in the Introduction, our study objective was to explore the potential contribution of CLIC1 to the pathophysiology of ESCC. Towards this, we first investigated the comparative expression of CLIC1 in ESCC (C) versus normal adjacent (N) tissues (≥5 cm distal to tumor) using immunohistochemistry in a cohort of 86 human ESCC tissue samples embedded as TMAs (Fig. 1A). In normal glandular areas, CLIC1 expression was detected in the cell nucleus of supra basal cells whereas stronger nuclear labelling was evident in ESCC cells along with some indications of membrane and cytoplasmic staining (Fig. 1B). Quantitative analysis of staining revealed that the overall expression of CLIC1 was significantly upregulated in ESCC tissues, compared with normal adjacent tissues (*P* < 0.001) (Fig. 1C). As independent confirmation of these data, the expression of CLIC1 in clinical ESCC specimens was further analyzed in eight pairs of fresh cancer tissues with matched adjacent normal tissues. Western blotting analyses of these samples showed the majority of cases the expression of CLIC1 protein was higher in ESCC tissues compared with their adjacent normal tissues (Fig. 1D). Furthermore, consistent with CLIC1 protein upregulation, RT-PCR-based measurements of CLIC1 mRNA levels showed increased expression in cancer versus healthy tissues (Fig. 1E). Notably, the expression of CLIC1 was also upregulated in ESCC cell lines (ECA109, KYSE30, KYSE70, KYSE150, KYSE520 and TE1), compared with normal human esophageal epithelial cells (HET-1A) (Fig. 1F).

Clinicopathological data was available for the cohort of 86 ESCC cases including 7 at clinical stage I, 40 at clinical stage II and 39 at clinical stage III, so we took the opportunity to evaluate associations with CLIC1 expression (Table 1). Actually, there are 96 ESCC cases in TMA samples, but only 86 of which have corresponding fresh tissues for further qPCR and Western blotting analysis, so the total number of ESCC cases reported here is 86. Imputing the CLIC1 expression levels determined from immunohistochemistry against clinicopathological data showed that CLIC1 expression was positively correlated with TNM stage with higher expression in advanced tumors (both stages II and III, *P* = 0.030 and 0.033, respectively). However, co-analysis of CLIC1 expression with other variables, gender, age, differentiation status and lymph node metastasis showed no significant association. Thus, CLIC1 is frequently upregulated in both ESCC tissues and cell lines compared to normal tissues. Moreover, the increased expression of CLIC1 in advanced disease stages suggests it may also participate in disease progression.

CLIC1 and the global ESCC proteome

To glean further clues regarding the functional contributions of CLIC1 to ESCC, we employed quantitative proteomics to identify differentially expressed proteins (DEPs) associated with CLIC1 knock-down. CLIC1 expressions in both lentiviral CLIC1 shRNA1 (shCLIC1-1) and shRNA2 (shCLIC1-2) knockdown cells were significantly decreased compared to negative control (Fig. 2A). Comparisons between negative control versus CLIC1 knockdown ECA109A ESCC cells identified a total of 5903 proteins, of which data for 5247 proteins were suitable for quantitative analysis. Between the two groups, a total of 183 proteins

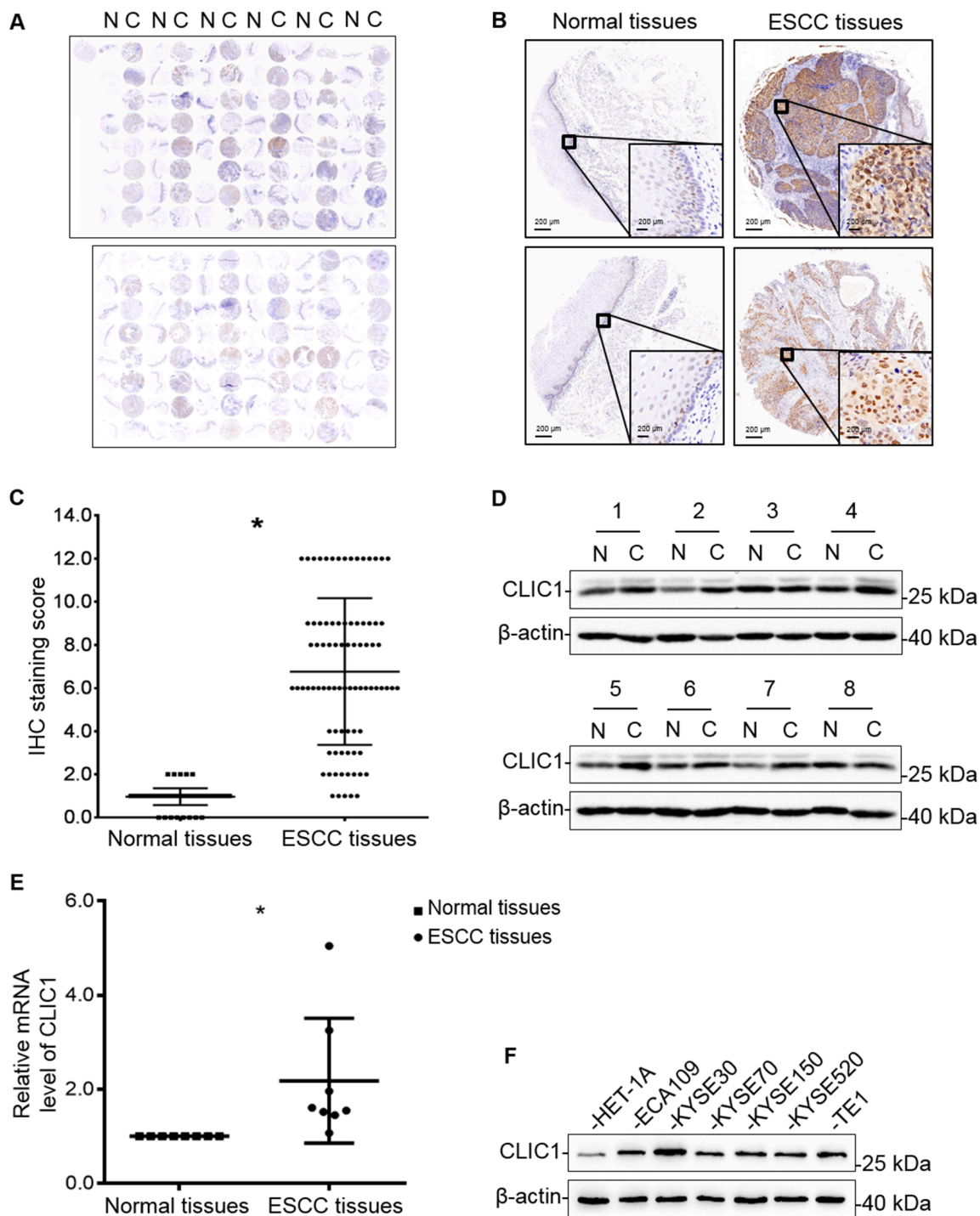


Fig. 1. CLIC1 expression is upregulated in ESCC versus normal tissues. **A.** Gross TMA images showing CLIC1 immunostaining of 86 ESCC and normal adjacent tissue samples. **B.** Representative images of CLIC1 and immunostaining from the cohort shown in (A). Positive (DAB) staining is denoted by the brown color with tissue structures highlighted by counterstaining with hematoxylin (light blue). **C.** CLIC1 expression determined by IHC in a cohort of 86 cases of normal and ESCC tissues. **D.** Western blotting of CLIC1 expression in fresh tissue extracts of eight ESCC tissues with paired normal tissues. β-actin was used as loading control. **E.** Relative CLIC1 mRNA expression in tissues from (C) as determined using RT-PCR. Mean ± SEM. n=3. *P < 0.05 (significantly different between two groups). **F.** CLIC1 expression determined by Western blotting as per against normal human esophageal epithelial cell (HET-1A) and ESCC cell lines (ECA109, KYSE30, KYSE70, KYSE150, KYSE520 and TE1).

were identified as DEPs, with 67 and 116 proteins being upregulated and downregulated between CLIC1 knockdown and the negative control groups, respectively (Fig. 2B). To further investigate the differences between the two groups, We supplemented these analyses using bioinformatic predictions to profile enriched processes and pathways using KEGG pathway and heat map analysis (Fig. 2C and 2D). Instructively,

these data collectively revealed an association between CLIC1 expression in ESCC and modulation of PI3K-Akt signaling. On this basis, we proceeded to further analyze the connection between CLIC1 expression and function in ESCC, particularly how it relates to oncogenic PI3K-Akt pathway activation.

Table 1
Relationship of clinicopathological characteristics of patient samples and expression of CLIC1 in ESCC.

Characteristics	Cases	Histochemistry score of CLIC1 in ESCC ^a	P value
Gender			
Male	65	6.939 ± 3.504	0.416 ^b
Female	21	6.238 ± 3.097	
Age			
≤64 ^c	51	6.804 ± 3.453	0.905 ^b
>64	35	6.714 ± 3.383	
TNM Stage			
I	7	4.000 ± 2.708	0.030 ^{d1}
II	40	7.100 ± 3.463	
III	39	6.923 ± 3.304	
Differentiation			
High	22	6.591 ± 3.686	0.946 ^e
Medium	52	6.865 ± 3.236	
Low	12	6.667 ± 3.869	
Lymph node metastasis			
No	44	7.000 ± 3.685	0.600 ^b
Yes	41	6.610 ± 3.097	

^a Data are presented as Mean ± SEM.

^b Student's *t*-test, a *P* value less than 0.05 was considered statistically significant.

^c The median age of the patients was 64.

^d Student's *t*-test, a *P* value less than 0.05 was considered statistically significant. ^{d1}TNM I vs TNM II. ^{d2}TNM I vs TNM III.

^e One-way ANOVA, a *P* value less than 0.05 was considered statistically significant.

Silencing of CLIC1 suppresses ESCC cell proliferation

To investigate the function of CLIC1 in ESCC cell lines, specific siRNAs targeting CLIC1 (siCLIC1) were used to suppress CLIC1 expression in the KYSE150 and TE1 cell lines previously shown to exhibit upregulated CLIC1 expression. Two independent CLIC1 targeting sequences (siCLIC1-1, siCLIC1-2) along with a negative control siRNA (siCtrl) were introduced into the cells by transient transfection for 48 h or 72 h and CLIC1 expression analyzed by Western blotting (Fig. 3A and 3B).

Further analysis using CCK-8 assays conducted daily over a period of 96 h showed that CLIC1 silencing with either siCLIC1-1 or siCLIC1-2 reduced the proliferative capacity of both KYSE150 and TE1 cell lines compared to the siCtrl-transfected cells (Fig. 3C). To further substantiate these data, we alternatively conducted colony formation assays to determine the effects of CLIC1 knockdown on ESCC cell proliferation. Representative images of these assays are shown in Fig. 3D with quantitation of colony numbers/growth area indicating that depleting CLIC1 significantly reduced the ability of both KYSE150 and TE1 cell lines to establish colonies (Fig. 3E). Collectively these results propose CLIC1 is involved in regulating ESCC proliferation.

CLIC1 promotes ESCC cell proliferation by regulating mTOR pathway

To explore whether CLIC1 promoted ESCC cell proliferation through effects on PI3K/Akt signaling, we next evaluated whether the expression and activity of key molecules in the PI3K/Akt/mTOR signaling pathway were affected by CLIC1 silencing. As before, two independent CLIC1-targeting sequences (siCLIC1-1, siCLIC1-2) and negative control siRNA (siCtrl) were introduced into KYSE150 and TE1 cell lines by transient transfection for 72 h. Western blotting analysis showed that the protein expression of p-Akt did not change significantly after CLIC1 silencing, while the protein expression of p-mTOR was decreased (Fig. 4A). These results suggest that CLIC1 does not directly affect PI3K/Akt but promotes phosphorylation activation of mTOR in ESCC cells. mTOR functions by forming two complexes: mTORC1 and mTORC2. The effects of CLIC1 silencing on the two complexes and their downstream signaling pathways were further explored. Western blotting analysis showed that CLIC1 silencing decreased the expression of p-4EBP1 protein, a key

downstream molecule of mTORC1, while p-S6K and p-S6 did not change significantly. In addition, the expression of Rictor, a key component of mTORC2, was also significantly decreased after CLIC1 silencing (Fig. 4B). In conclusion, CLIC1 can promote the activation of both mTORC1 and mTORC2 in ESCC by upregulating p-mTOR, Rictor and 4EBP1 expression.

CLIC1 promotes ESCC cell proliferation through mTOR

Since activation of mTOR pathway promotes cell proliferation of tumor cells [35], and we next analyzed whether the promotion of CLIC1 in ESCC cells proliferation was involved in mTOR signaling. To test whether CLIC1 promotes cell proliferation through mTOR, we cotransfected cells with ORF (encoding mTOR) with CLIC1-targeting sequences. The inhibition in cell proliferation induced by CLIC1 knockdown were rescued by mTOR overexpression (Fig. 5A–D), suggesting that CLIC1 promotes cell proliferation through mTOR signaling.

Discussion

Esophageal cancer (EC) is a digestive tract cancer associated with significant morbidity, poor prognosis and high mortality. EC has a high incidence in Asian and Middle Eastern countries where it constitutes the sixth most lethal disease with a five-year survival of about 30% [39]. Over 90% of EC patients in China are diagnosed with ESCC, the majority subtype with poor prognosis and high mortality [11]. In this study, we investigated the expression of CLIC1 in a cohort of Chinese ESCC patients using IHC, Western blotting and qPCR analyses. These experiments established that elevated CLIC1 expression commonly occurs in ESCC tissues and, as discerned from our IHC data, this predominantly results from increased CLIC1 expression in the cancer cells themselves. Moreover, co-variate analyses with the available clinicopathological data showed that higher expression of CLIC1 was associated with more advanced disease stages corresponding with TNM stages II and III although CLIC1 expression was not associated with pathological grading. Thus, ESCC joins the growing list of cancers reported to display elevated CLIC1 expression [10–16] which has further been correlated with different aspects of tumour development in various cancers, including lymph node metastasis, pathological staging, and poor prognosis, for example, as reported in gallbladder, serious epithelial ovarian and oral squamous cell carcinomas [12,40,41].

From a functional viewpoint, CLIC1 has been shown to influence oncogenic behaviours in cancer cells through distinct pathways, including the MAPK/ERK signalling in prostate cancer cells, ROS-mediated p38 MAPK signalling in gastric cancer, ROS/ERK pathway in colon cancer cells, and integrins/ERK pathway in oral squamous cell carcinoma [42]. Down-regulation of CLIC1 inhibits cell migration and invasion in colonic, prostate, gastric cancer cells [17,43]. In our analysis of ESCC, based on assays of cell proliferation and cell viability, silencing of CLIC1 significantly reduced the cell growth capacity of two ESCC cell lines displaying elevated CLIC1 expression. These findings align with the proposed oncogenic role of CLIC1 established in other cancer types. However, our findings appear to contrast the report from Kobayashi et al. who showed that depletion of CLIC1 enhanced cell migration and invasion in ESCC cells, acting through the MAPK/ERK pathway in ESCC [44]. Interestingly, they also revealed that very strong or alternatively weak expression of CLIC1 was associated with poor prognosis in ESCC patients [44]. However, these divergent findings are not mutually exclusive since it is possible that CLIC1 participates in functional switching behaviour in ESCC between proliferative and migratory states, although more work is required to address this hypothesis. Notwithstanding this point, our findings highlight that CLIC1 overexpression is a feature of ESCC, proposing CLIC1 as a potential biomarker therapeutic target.

The highly conserved serine/threonine protein kinase mTOR plays a key role in regulating cells growth and metabolism. Many prior studies

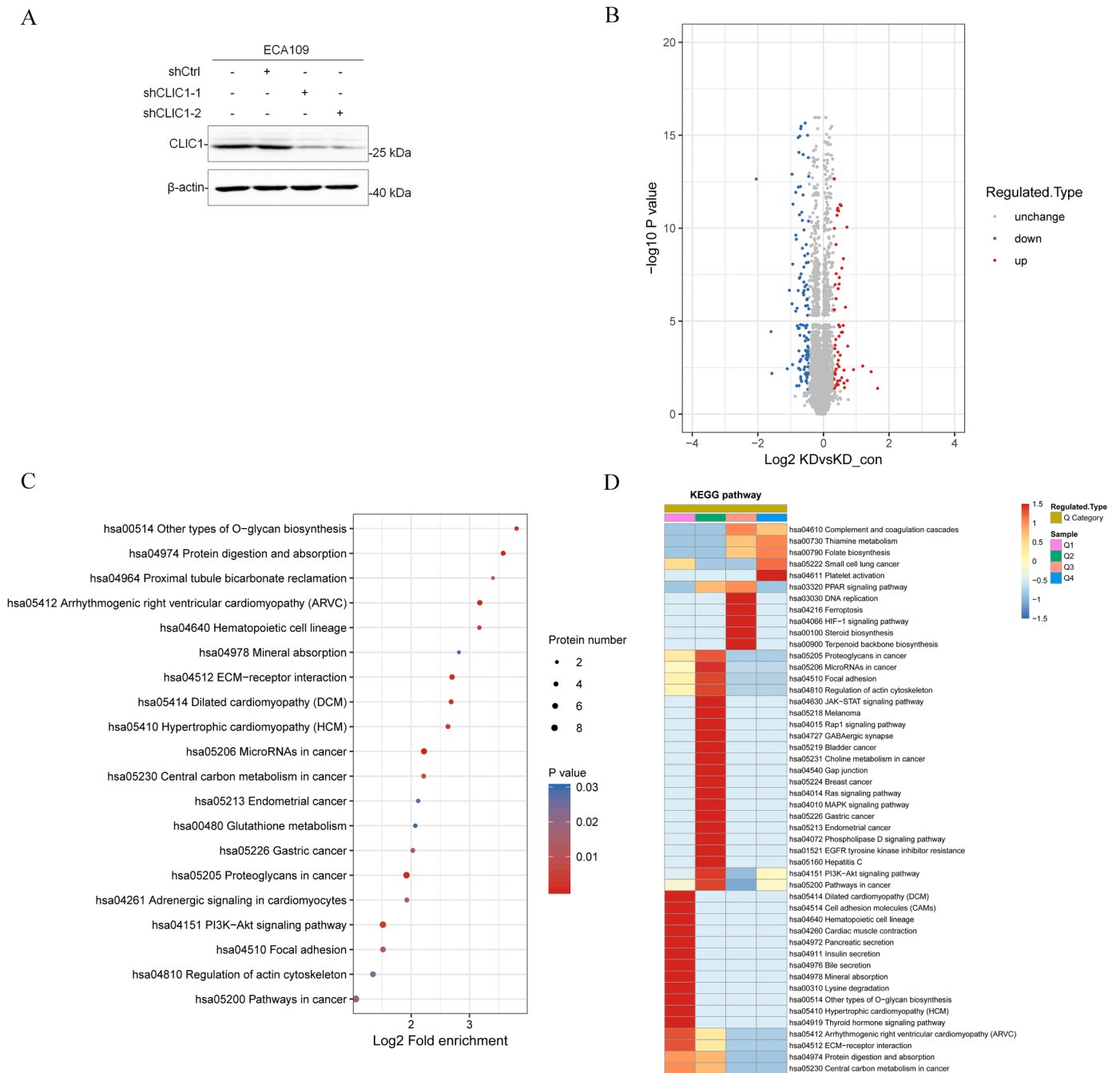


Fig. 2. Differentially expressed proteins (DEPs) and signalling pathways analysed between ESCC cells with or without CLIC1 knockdown. **A.** Western blotting analysis of lentiviral CLIC1 shRNA knockdown for proteomic analysis. **B.** Volcano plots of DEPs comparing the upregulated and downregulated proteins in ECA109 with CLIC1 knockdown. **C.** KEGG pathway enrichment bubble plot of DEPs in ESCC with CLIC1 knockdown. **D.** A comprehensive heatmap for cluster analysis of the enrichment patterns of KEGG pathways.

have shown that abnormal activation of mTORC1 promotes tumorigenesis through 4EBP1/eIF4E mediated translation of target genes [45–47]. mTORC2 has also been found to be essential for cell cycle progression and cell growth in both PTEN-deficient human prostate cancer cell lines and mouse prostate cancer models [48–50]. Our study showed the regulation of CLIC1 in activation of mTOR signaling. The expression of p-4EBP1 and Rictor which are components of mTORC1 and mTORC2, respectively, are up-regulated in response to CLIC1 knockdown in ESCC cells. It indicates the upstream regulation of CLIC1 in mTOR activation.

Our study now connects CLIC1 expression with mTOR signaling in ESCC where CLIC1 expression promoted the phosphorylation and

activation of mTOR along with Rictor expression, thereby promoting ESCC cell proliferation through the mTORC1/4EBP1 and mTORC2 signaling pathways (Fig. 6).

Author contributions

X.Y.Liu and X.Y.Li conceived, designed the project and wrote the manuscript. H.W.G and C.F designed and performed most of the experiment. Z.R.S, X.F and Y.Q.X performed some cellular experiments. G.L and C.L analyzed some immunohistochemistry data. R.F.T and X.D.Z provided assistance for revision of the manuscript.

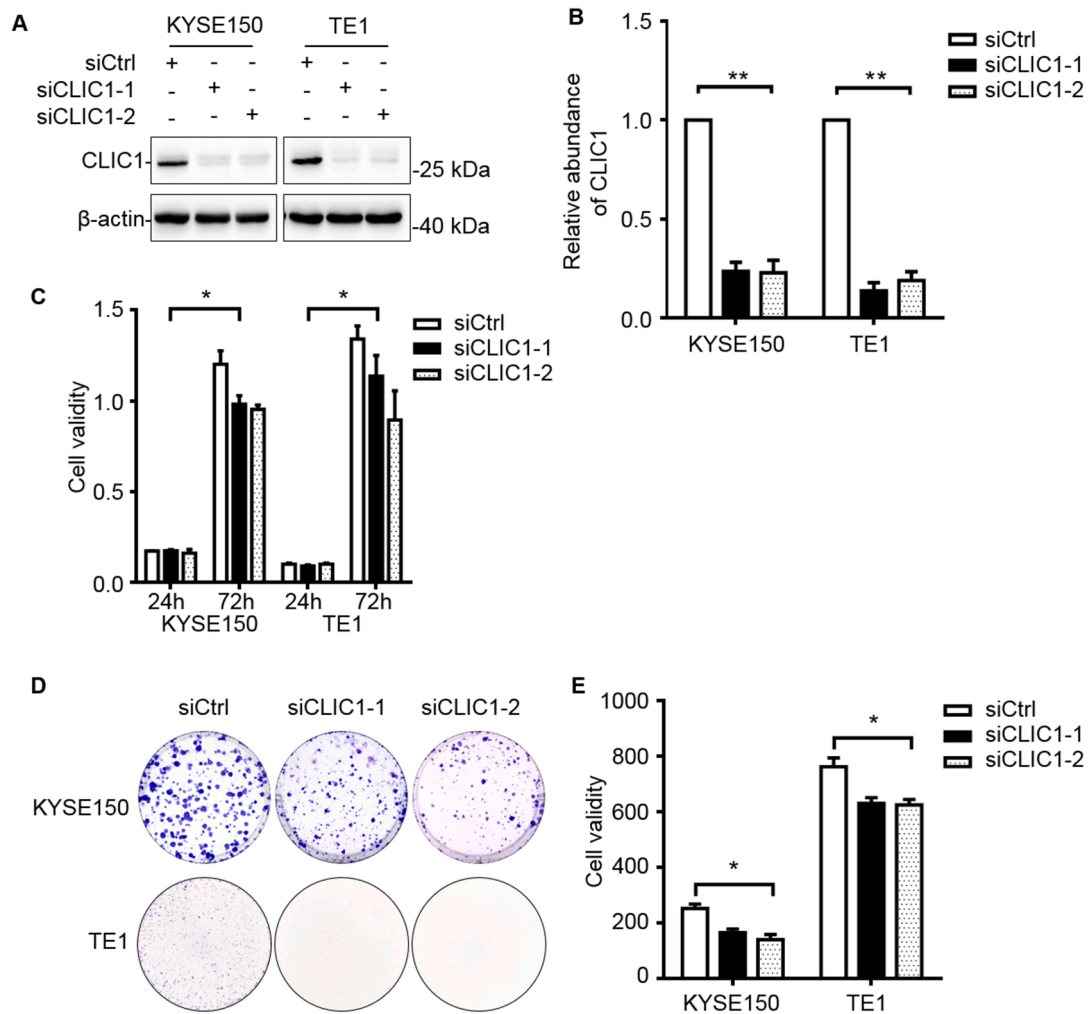


Fig. 3. Silencing of CLIC1 suppresses cell proliferation in ESCC cells. A-B. Western blotting analyses of CLIC1 expression in siCtrl or siCLIC1 transfected KYSE150 and TE1 cells. C. CCK-8 assays of cell proliferation in ESCC cells transfected with siCtrl or siCLIC1s. Mean ± SEM. n=3. **P* < 0.05 (significantly different between siCtrl and siCLIC1s). D-E. Down-regulation of CLIC1 reduced the mean colony number in the colony formation assay. Mean ± SEM. n=3. **P* < 0.05 (significantly different between siCtrl and siCLIC1s).

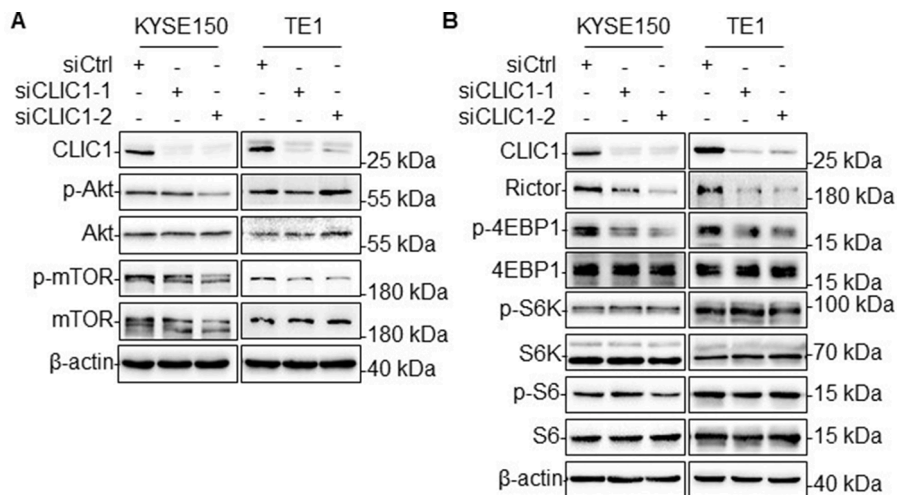


Fig. 4. CLIC1 knockdown inhibits mTOR and its downstream pathways in ESCC cells. A-B. CLIC1 siRNA knockdown in KYSE150 and TE1 cells. The indicated proteins were detected by Western blotting, and β-actin was used as loading control.

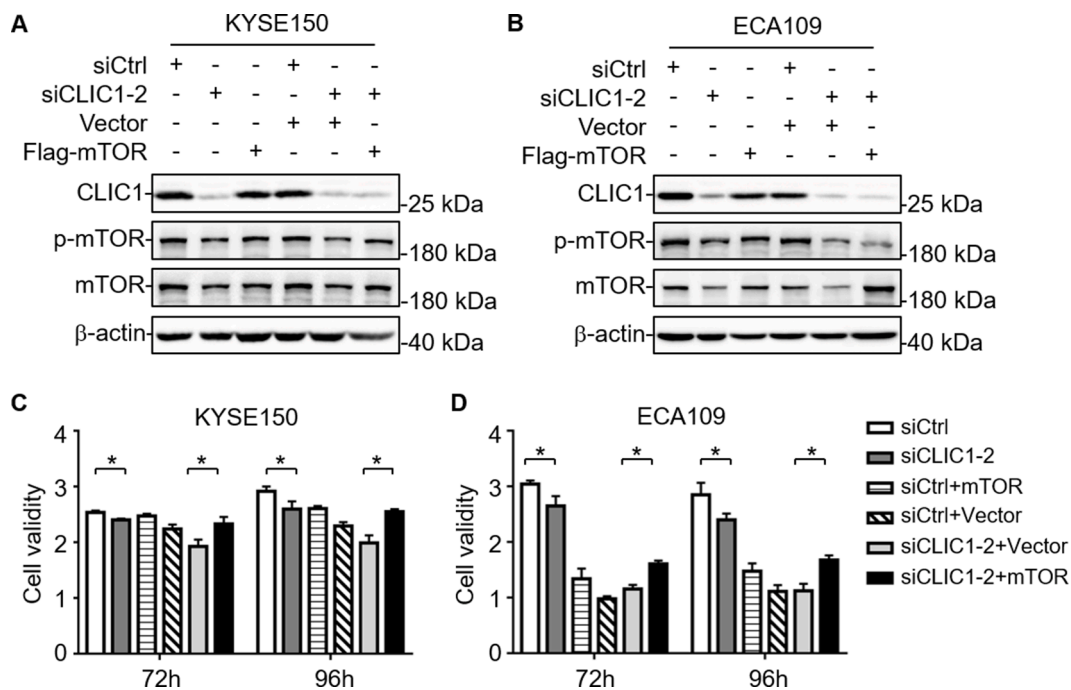


Fig. 5. Overexpression of mTOR can rescue ESCC cells from CLIC1 mediated inhibition of cell proliferation. A-B. KYSE150 and ECA109 cells were transfected with siCtrl or si-CLIC1 and then transfected with vector pcDNA3.1 or plasmid for m-TOR overexpression. The indicated proteins were detected by Western blotting, and β -actin was used as loading control. C-D. CCK-8 assays of cell proliferation in A and B. Mean \pm SEM. $n=3$. $*P < 0.05$.

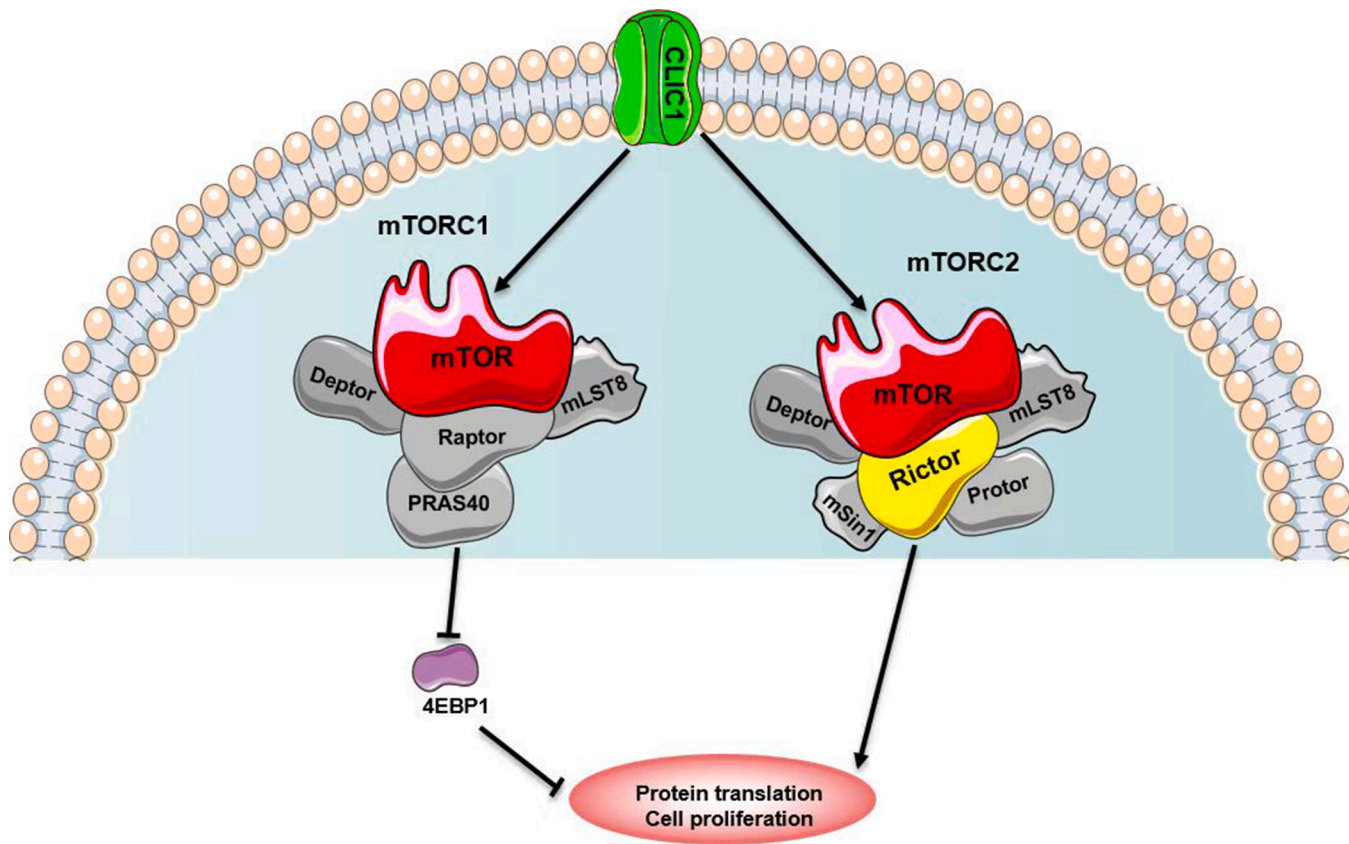


Fig. 6. CLIC1 promotes cell proliferation of ESCC by upregulating mTOR signaling pathway.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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