European Review for Medical and Pharmacological Sciences 2020; 24: 5429-5435

CSE1L promotes proliferation and migration in oral cancer through positively regulating MITF

Y.-S. WANG, C. PENG, Y. GUO, Y. LI

Department of Dental, Second Hospital Affiliated to Tianjin Medical University, Tianjin, China

Abstract. – OBJECTIVE: CSE1L (human chromosomal segregation 1-like) is reported to be able to affect cell apoptosis, invasiveness, and migration. The purpose of this study was to uncover the regulatory effects of CSE1L on cell phenotypes of oral cancer and the underlying mechanism.

MATERIALS AND METHODS: CSE1L levels in oral cancer cells were determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and Western blot. CSE1L overexpression and knockdown models were constructed in CAL-27 and HN6 cells, respectively. Changes in proliferative and migratory abilities in oral cancer cells affected by CSE1L and microphthalmia-associated transcription factor (MITF) were assessed by cell counting kit-8 (CCK-8), 5-Ethynyl-2'-deoxyuridine (EdU) and wound healing assay. Meanwhile, potential influences of CSE1L and MITF on relative levels of E-cadherin and Vimentin in oral cancer cells were detected. Finally, regulatory effects of CSE1L and MITF on the Akt/mTOR pathway were evaluated by detecting expression levels of p-Akt, Akt, p-mTOR, and mTOR.

RESULTS: CSE1L was upregulated in oral cancer cells. Knockdown of CSE1L in HN6 cells attenuated proliferative and migratory abilities, as well as downregulated Vimentin and upregulated E-cadherin. Overexpression of CSE1L in CAL-27 cells yielded the opposite results. MIFT level was positively regulated by CSE1L. Overexpression of MITF partially reversed regulatory effects of CSE1L on proliferative ability of oral cancer cells. Moreover, silence of CSE1L suppressed the Akt/mTOR pathway, which was reversed by overexpression of MITF.

CONCLUSIONS: CSE1L promotes the proliferative and migratory abilities in oral cancer cells by positively regulating MITF, thus activating the Akt/mTOR pathway.

Key Words:
Oral cancer, CSE1L, MITF, Akt/mTOR pathway.

Introduction

Oral cancer is a highly differentiated malignancy originating in the head and neck. Oral cancer is prone to infiltrate to cervical lymph nodes, while its rate of distant metastasis is relatively low. Poor sensitivities of oral cancer to chemotherapy and radiotherapy lead to low response of anti-tumor treatment[1,2]. Uncontrolled primary lesions are the major cause of death related to oral cancer. It is estimated that the 5-year survival of oral cancer is 50-60%[3]. Therefore, it is necessary to develop effective strategies for clinical treatment of oral cancer.

Human chromosomal segregation 1-like (CSE1L), also known as cellular apoptosis susceptibility (cAS), was initially discovered in breast cancer cells. CSE1L locates on human chromosome 20q13, which is homologous with CSE1. CSE1L is found to be upregulated in multiple types of cancer cells[4]. As a nuclear transport factor, CSE1L participates in the regulation of cell apoptosis[5], proliferation[6], chromosome aggregation[7], microvesicle formation[8,9], tumor metastasis[6], and embryonic development in the early phase[10].

Microphthalmia-associated transcription factor (MITF) locates on human chromosome 3p12.3-14.1 and encodes the gene containing 419 amino acids with 46 kDa. It is tissue-specific, containing a dimer structure of bHLH-Zip[11]. Through the bHLH-Zip domain, MITF recognizes E-box sequences (CATGTG) in the promoter regions of target gene, thus initiating the transcription of downstream genes[12]. Besides, MITF exerts an important role in the development of melanocytes, retinal pigment epithelial cells, hypertrophic cells, and osteoclasts, and it also contributes to maintain cell morphology[13]. In glioma, CSE1L affects disease progression by targeting MITF[14]. Thus, the purpose of this study was to uncover the potential influences of CSE1L and MITF on the progression of oral cancer.

Materials and Methods

Cell Culture

Oral cancer cells (HN4, HN6, CAL-27, and SCC-4) were purchased from American Type

Corresponding Authors: Cheng Peng, MD; e-mail: peng_cheng2013@163.com

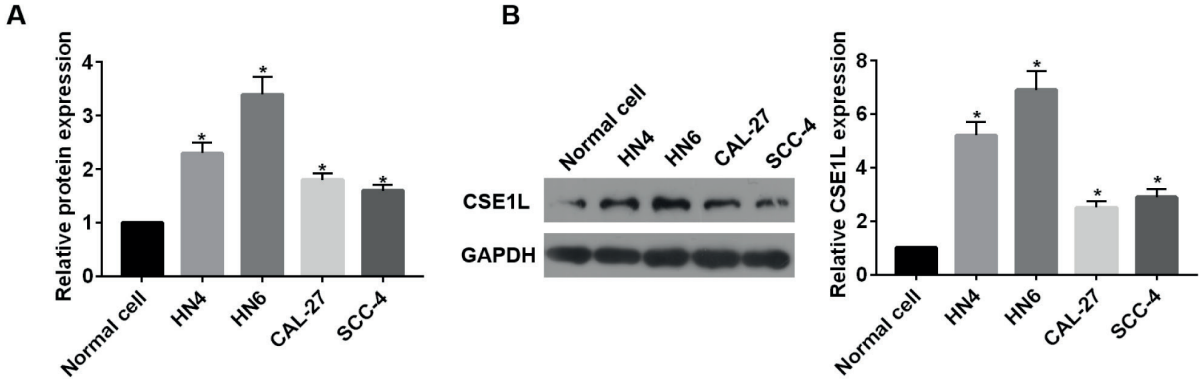

Figure 1. CSE1L is upregulated in oral cancer cells. The mRNA (**A**) and protein level (**B**) of CSE1L in normal cells and oral cancer cells (HN4, HN6, CAL-27, and SCC-4).

Culture Collection (ATCC; Manassas, VA, USA). Then, the cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% penicillin-streptomycin. Medium was regularly replaced, and cell passage was conducted using trypsin.

RNA Extraction and Quantitative Real-Time RCR (qRT-PCR)

TRIzol (Invitrogen, Carlsbad, CA, USA) was applied for isolating cellular RNA, which was quantified using a spectrometer. Next, RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using the PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). After that, SYBR Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan) was utilized for qRT-PCR. Primer sequences are listed as follows: CSE1L-F: 5′-TTTTGAGTTACCCGAAGA-3′; CSE1L-R: 5′-TTGTGAAGTGACTGTGCC-3′; GAPDH-F: 5′-GTCAGCCGCATCTTCTTTTG-3′; glyceraldehyde 3-phosphate dehydrogenase (GAPDH)-R: 5′-GCGCCCAATACGACCAAATC-3′.

Transfection

Pre-designed siRNA sequences targeted to CSE1L (5′-GCATGATCCTGTAGGTCAA-3′) were synthesized by the GenePharma Company (Shanghai, China) and transfected into HN6 cells. In the meantime, siRNA-NC (5′-UUCUCCGAACGUGUCACGUTT-3′) was used as a negative control. Then, cell transfection was conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Western Blot

Cells were lysed using cell lysis buffer (Qincheng Biotech, Shanghai, China; Cat no: QC25-05099), shaken on ice for 30 min, and centrifuged at 14,000 x g for 15 min at 4°C. Total protein concentration was calculated by bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL, USA). Protein samples were separated using a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and then loaded on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 h. Next, membranes reacted with primary and secondary antibodies for indicated time. Band exposure and analyses were finally conducted. The gray value was analyzed using ImageJ software (Version 1.38; National Institutes of Health, Bethesda, MA, USA).

Cell Counting Kit-8 (CCK-8)

Cells were inoculated in the 96-well plate (3×10^3 cells per well) and added with 10 μL CCK-8 (Dojindo Laboratories, Kumamoto, Japan) reagent per well for 4 h culture. Absorbance (A) at 450 nm was measured by a microplate reader.

5-Ethynyl-2′-Deoxyuridine (EdU) Assay

Cells were inoculated into a 6-well plate and labeled with 50 μM EdU reagent for 2 h. After washing with phosphate-buffered saline (PBS), cells were fixed in 50 μL of fixation buffer, decolored with 2 mg/mL glycine, and permeated with 100 μL of penetrant. After PBS washing once, cells were stained with 100 μL of 4',6-diamidino-2-phenylindole (DAPI) in the dark for 30 min. Ultimately, EdU-positive cells, DA-

PI-labeled cells, and their merged images were captured under a fluorescent microscope.

Wound Healing Assay

All instruments were required for 30-min ultraviolet radiation, including the ruler, marker pen, and 200 μL pipette. Two lines with 0.5-1 cm interval in the middle were depicted on the back of a 6-well plate using a marker pen. The cells were inoculated in a 6-well plate at 5×10^5 cells/well, and an artificial wound was created in the confluent cell monolayer using a 200 μL pipette tip. Finally, wound closure was captured at 0 and 24 h, respectively.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 16.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Data were expressed as mean ± SD (standard deviation). Differences between two groups were analyzed by the *t*-test. $p<0.05$ indicated the significant difference.

Results

CSE1L Was Upregulated in Oral Cancer Cells

Compared with normal cells, CSE1L was upregulated in oral cancer cells at both mRNA and protein levels (Figure 1A, 1B). In particular, CSE1L presented the highest abundance in HN6 cells and the lowest one in CAL-27 cells among the four tested oral cancer cell lines, so HN6 and CAL-27 cells were selected for the following experiments.

CSE1L Promoted Proliferative Ability in Oral Cancer Cells

To uncover the biological role of CSE1L in oral cancer, CSE1L knockdown and overexpression models were constructed in HN6 and CAL-27 cells, respectively. Transfection of si-CSE1L effectively downregulated protein level of CSE1L in HN6 cells, and transfection of pcDNA-CSE1L markedly upregulated CSE1L in CAL-27 cells (Figure 2A). In HN6 cells transfected with si-CSE1L, viability, and EdU-positive ratio were remarkably reduced. On the contrary, viability and EdU-positive ratio markedly increased in CAL-27 cells overexpressing CSE1L (Figure 2B, 2C).

CSE1L Promoted Migratory Ability in Oral Cancer Cells

Wound healing assay revealed that knockdown of CSE1L in HN6 cells greatly attenuated migratory ability, while overexpression of CSE1L in CAL-27 cells yielded the opposite trend (Figure 3A). Western blot analyses uncovered that silence of CSE1L upregulated E-cadherin and downregulated Vimentin, and the opposite trends were observed after overexpression of CSE1L (Figure 3B). It can be concluded that CSE1L stimulates oral cancer cells to migrate.

CSE1L Promoted Proliferative Ability in Oral Cancer Cells Via Regulating MITF

Of note, protein level of MITF was downregulated in HN6 cells transfected with si-CSE1L (Figure 4A). The decreased viability in HN6 cells with CSE1L knockdown was partially reversed by overexpression of MITF (Figure 4B). Moreover, overexpression of MITF abolished the reduced EdU-positive ratio in HN6 cells transfected with si-CSE1L (Figure 4C). Hence, MITF was responsible for the proliferation phenotype of oral cancer regulated by CSE1L.

CSE1L Activated the Akt/mTOR Pathway in Oral Cancer Through Positively Regulating MITF

It is shown that the ratios of p-Akt/Akt and p-mTOR/mTOR were markedly reduced in HN6 cells transfected with si-CSE1L, indicating that knockdown of CSE1L suppressed the Akt/mTOR pathway. Nevertheless, the inactivated Akt/mTOR pathway was partially abolished by overexpression of MITF (Figure 5). As a result, it can be concluded that CSE1L activates the Akt/mTOR pathway in oral cancer by positively regulating MITF.

Discussion

Oral cancer, a prevalent tumor in the head and neck, has become the sixth leading cancer globally. The mortality of oral cancer is up to 70%. More seriously, impaired physiological functions of speech, swallowing, and chewing because of oral cancer severely influence life quality of affected patients[15]. Epidemiological investigations[16] have demonstrated that the incidence of oral cancer presents a younger trend, posing a great burden on the whole society.

Chromosome 20q13 tends to be amplified in various cancer tissues[17]. Amplification state of chromosome 20q13 is closely linked to cancer progression[18,19]. It is reported that the amplification of chromosome 20q predicts invasiveness,

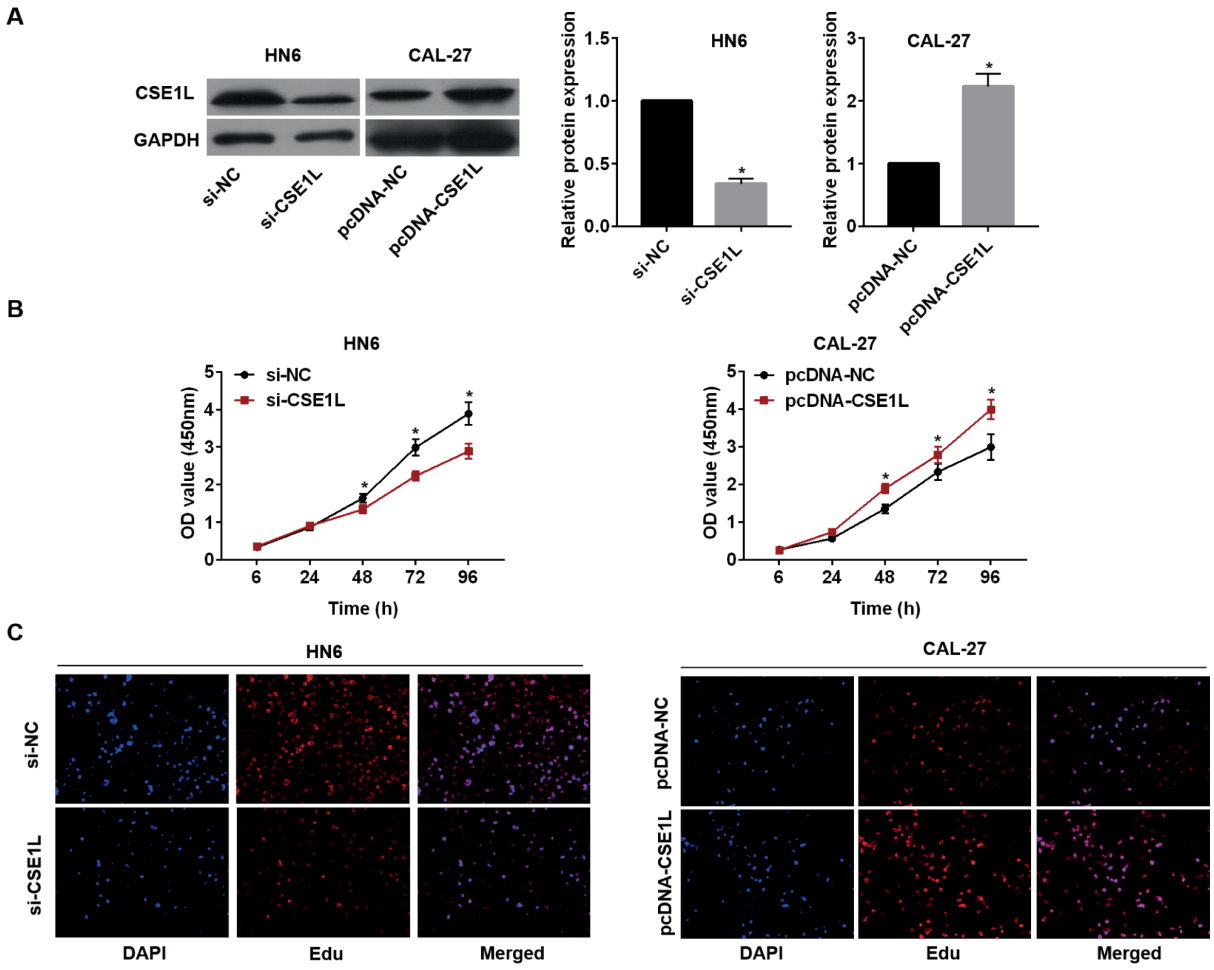

Figure 2. CSE1L promotes proliferative ability in oral cancer cells. HN6 cells were transfected with si-NC or si-CSE1L. CAL-27 cells were transfected with pcDNA-NC or pcDNA-CSE1L. **A,** Protein level of CSE1L, **B,** Cell viability, **C,** EdU-positive cells (magnification 40×).

metastasis, and poor prognosis of cancer[20,21]. CSE1L locates on human chromosome 20q13. In colon cancer cells, knockdown of CSE1L attenuates clonality and induces apoptosis[22]. Yuksel et al[23] pointed out that cytoplasmic level of CSE1L is correlated with axillary lymph node metastasis in breast cancer patients. Besides, Lorenzato et al[24] suggested that Akt activation triggers nuclear aggravation of CSE1 in ovarian cancer cells, thereafter, affecting the transportation of carcinogenic signaling. In this paper, it was discovered that CSE1L was upregulated in oral cancer cells. Knockdown of CSE1L attenuated proliferative and migratory abilities in oral cancer.

MITF presents at least 9 different promoter-exon structures. The first exon and different promoters determine the specific subtype of MITF, while downstream exons are the same. Different subtypes of MITF is expressed in different types of cells[13,25]. MITF is of significance in the development and differentiation of certain cells in multiple organs[25]. Moreover, Bera et al[10] introduced the first MITF-mutant mouse from offsprings of mice undergoing X-ray radiation. So far, over 24 spontaneous and induced allele mutation sites of MITF have been discovered. Li et al[26] has suggested that knockdown of CSE1L alleviates the growth and metastasis of gastric cancer *via* positively regulating MITF. In this paper, overexpression of MITF reversed the inhibited proliferative ability in oral cancer cells with CSE1L knockdown. Furthermore, the suppressed Akt/mTOR pathway in oral cancer cells transfected with si-CSE1L was abolished by overexpressed MITF. To sum up, CSE1L was upregulated in oral cancer, and it promoted the proliferative and migratory

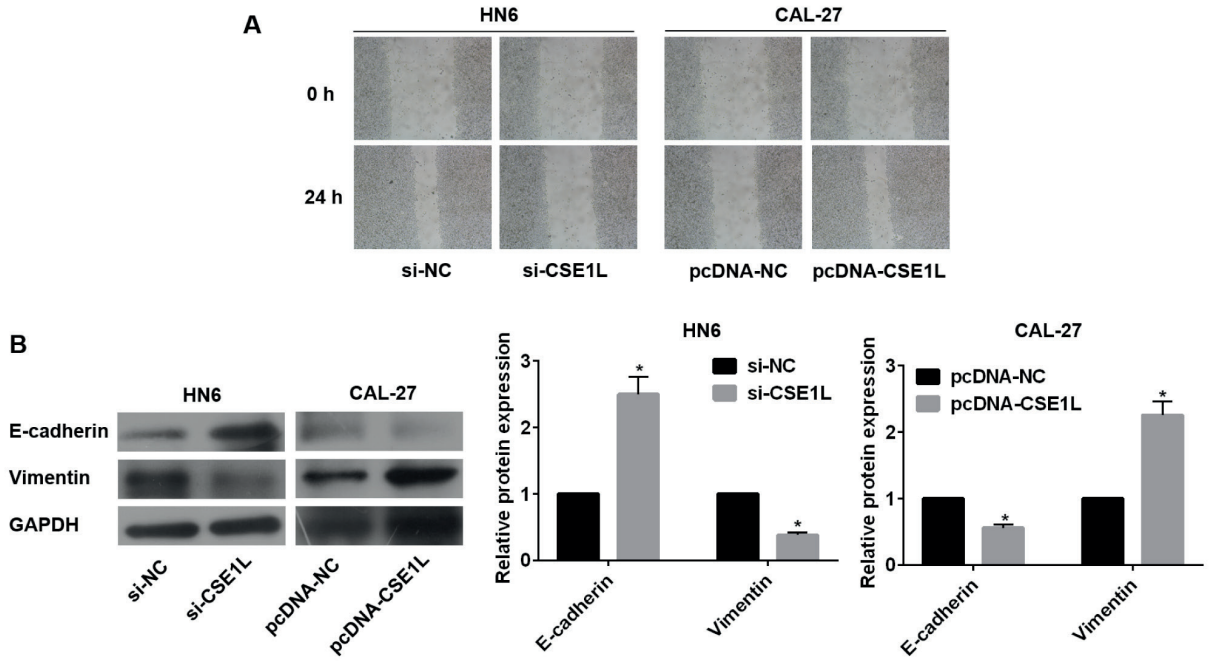

Figure 3. CSE1L promotes migratory ability in oral cancer cells. HN6 cells are transfected with si-NC or si-CSE1L. CAL-27 cells are transfected with pcDNA-NC or pcDNA-CSE1L. **A,** Percentage of wound healing (magnification 10×), **B,** Protein levels of E-cadherin and Vimentin.

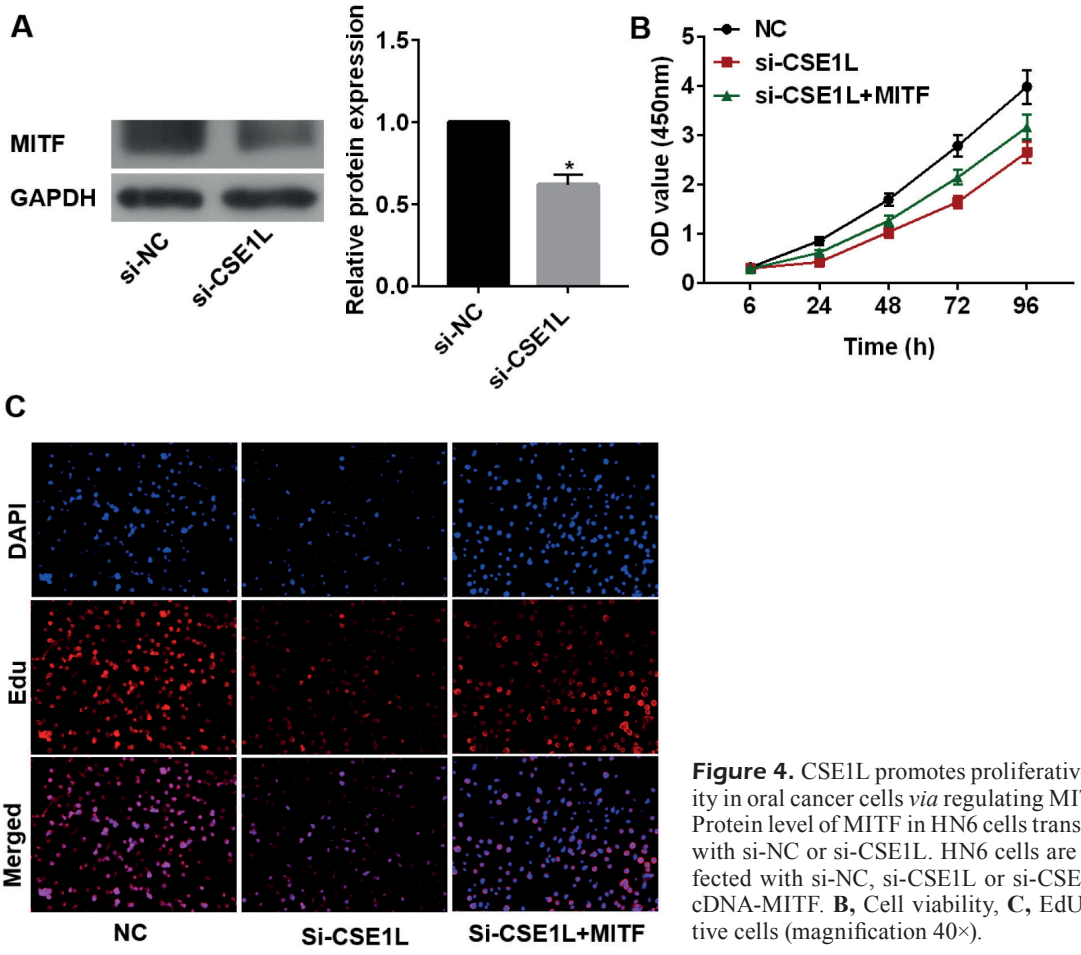

Figure 4. CSE1L promotes proliferative ability in oral cancer cells *via* regulating MITF. **A,** Protein level of MITF in HN6 cells transfected with si-NC or si-CSE1L. HN6 cells are transfected with si-NC, si-CSE1L or si-CSE1L+pcDNA-MITF. **B,** Cell viability, **C,** EdU-positive cells (magnification 40×).

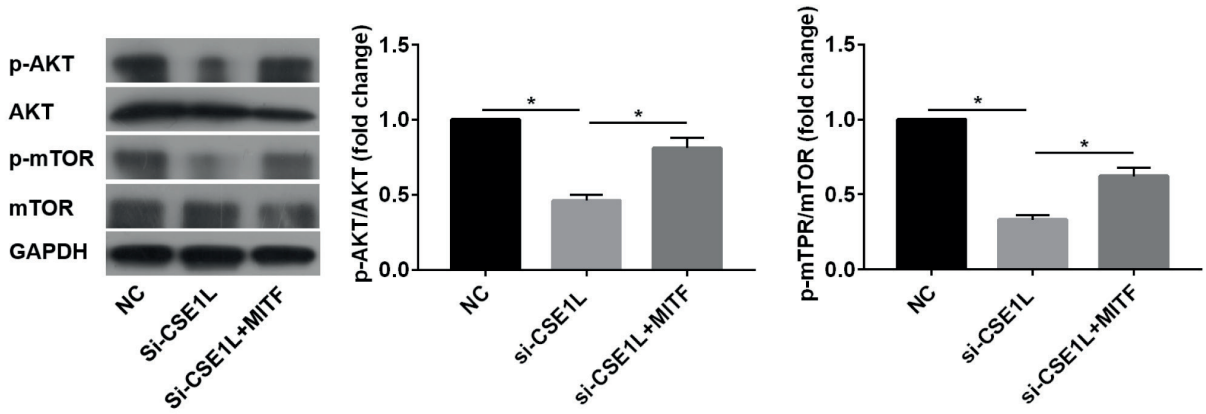

Figure 5. CSE1L activates the Akt/mTOR pathway in oral cancer through MITF. HN6 cells are transfected with si-NC, si-CSE1L or si-CSE1L+pcDNA-MITF. Protein levels of p-Akt, Akt, p-mTOR and mTOR. Ratios of p-Akt/Akt and p-mTOR/mTOR.

abilities in oral cancer by regulating MITF to activate the Akt/mTOR pathway. The findings provide novel directions in the clinical treatment of oral cancer.

Conclusions

Altogether the above data revealed that CSE1L promotes the proliferative and migratory abilities in oral cancer cells by positively regulating MITF, thus activating the Akt/mTOR pathway.

Conflict of Interests

The Authors declare that they have no conflict of interests.

References

1) Nagler R, Weizman A, Gavish A. Cigarette smoke, saliva, the translocator protein 18kDa (TSPO) and oral cancer Oral Dis. 2019 Aug 20. doi: 10.1111/odi.13178. [Epub ahead of print].
2) Azimi S, Ghorbani Z, Tennant M, Kruger E, Safiaghdam H, Rafieian N. Population survey of knowledge about oral cancer and related factors in the capital of Iran. J Cancer Educ 2019; 34: 116-123.
3) Salaun B, Coste I, Rissoan MC, Lebecque SJ, Renno T. TLR3 can directly trigger apoptosis in human cancer cells. J Immunol 2006; 176: 4894-4901.
4) Lee WR, Shen SC, Wu PR, Chou CL, Shih YH, Yeh CM, Yeh KT, Jiang MC. CSE1L Links cAMP/PKA and Ras/ERK pathways and regulates the expressions and phosphorylations of ERK1/2, CREB, and MITF in melanoma cells. Mol Carcinog 2016; 55: 1542-1552.
5) Brinkmann U, Brinkmann E, Pastan I. Expression cloning of cDNAs that render cancer cells resistant to Pseudomonas and diphtheria toxin and immunotoxins. Mol Med 1995; 1: 206-216.
6) Tai CJ, Hsu CH, Shen SC, Lee WR, Jiang MC. Cellular apoptosis susceptibility (CSE1L/CAS) protein in cancer metastasis and chemotherapeutic drug-induced apoptosis. J Exp Clin Cancer Res 2010; 29: 110.
7) Scherf U, Pastan I, Willingham MC, Brinkmann U. The human CAS protein which is homologous to the CSE1 yeast chromosome segregation gene product is associated with microtubules and mitotic spindle. Proc Natl Acad Sci U S A 1996; 93: 2670-2674.
8) Liao CF, Lin SH, Chen HC, Tai CJ, Chang CC, Li LT, Yeh CM, Yeh KT, Chen YC, Hsu TH, Shen SC, Lee WR, Chiou JF, Luo SF, Jiang MC. CSE1L, a novel microvesicle membrane protein, mediates Ras-triggered microvesicle generation and metastasis of tumor cells. Mol Med 2012; 18: 1269-1280.
9) Xu R, Greening DW, Rai A, Ji H, Simpson RJ. Highly-purified exosomes and shed microvesicles isolated from the human colon cancer cell line LIM1863 by sequential centrifugal ultrafiltration are biochemically and functionally distinct. Methods 2015; 87: 11-25.
10) Bera TK, Bera J, Brinkmann U, Tessarollo L, Pastan I. Cse1l is essential for early embryonic growth and development. Mol Cell Biol 2001; 21: 7020-7024.
11) Hodgkinson CA, Moore KJ, Nakayama A, Steingrimsson E, Copeland NG, Jenkins NA, Arnheiter H. Mutations at the mouse microphthalmia locus are associated with defects in a gene encoding a novel basic-helix-loop-helix-zipper protein. Cell 1993; 74: 395-404.
12) Meadows NA, Sharma SM, Faulkner GJ, Ostrowski MC, Hume DA, Cassady AI. The expression of Clcn7 and Ostm1 in osteoclasts is coregulated by microphthalmia transcription factor. J Biol Chem 2007; 282: 1891-1904.

13) Shibahara S, Takeda K, Yasumoto K, Udono T, Watanabe K, Saito H, Takahashi K. Microphthalmia-associated transcription factor (MITF): multiplicity in structure, function, and regulation. J Investig Dermatol Symp Proc 2001; 6: 99-104.

14) Lee WR, Shen SC, Wu PR, Chou CL, Shih YH, Yeh CM, Yeh KT, Jiang MC. CSE1L links cAMP/PKA and Ras/ERK pathways and regulates the expressions and phosphorylations of ERK1/2, CREB, and MITF in melanoma cells. Mol Carcinog 2016; 55: 1542-1552.

15) Achalli S, Madi M, Babu SG, Shetty SR, Kumari S, Bhat S. Sialic acid as a biomarker of oral potentially malignant disorders and oral cancer. Indian J Dent Res 2017; 28: 395-399.

16) Bregman JA. The oral cancer epidemic. Today's FDA 2016; 28: 32-33, 35.

17) Bernaciak J, Wisniowiecka-Kowalnik B, Castaneda J, Kutkowska-Kazmierczak A, Nowakowska B. A novel de novo 20q13.11q13.12 microdeletion in a boy with neurodevelopmental disorders - case report. Dev Period Med 2017; 21: 91-94.

18) Chen W, Hu XT, Shi QL, Zhang FB, He C. Knockdown of the novel proteasome subunit Adrm1 located on the 20q13 amplicon inhibits colorectal cancer cell migration, survival and tumorigenicity. Oncol Rep 2009; 21: 531-537.

19) Hsu PY, Hsu HK, Hsiao TH, Ye Z, Wang E, Profit AL, Jatoi I, Chen Y, Kirma NB, Jin VX, Sharp ZD, Huang TH. Spatiotemporal control of estrogen-responsive transcription in ERα-positive breast cancer cells. Oncogene 2016; 35: 2379-2389.

20) Postma C, Terwischa S, Hermsen MA, van der Sijp JR, Meijer GA. Gain of chromosome 20q is an indicator of poor prognosis in colorectal cancer. Cell Oncol 2007; 29: 73-75.

21) Wullich B, Riedinger S, Brinck U, Stoeckle M, Kamradt J, Ketter R, Jung V. Evidence for gains at 15q and 20q in brain metastases of prostate cancer. Cancer Genet Cytogenet 2004; 154: 119-123.

22) Pimiento JM, Neill KG, Henderson-Jackson E, Eschrich SA, Chen DT, Husain K, Shibata D, Coppola D, Malafa MP. Knockdown of CSE1L gene in colorectal cancer reduces tumorigenesis in vitro. Am J Pathol 2016; 186: 2761-2768.

23) Yuksel UM, Dilek G, Dogan L, Gulcelik MA, Berberoglu U. The relationship between CSE1L expression and axillary lymph node metastasis in breast cancer. Tumori 2015; 101: 194-198.

24) Lorenzato A, Biolatti M, Delogu G, Capobianco G, Farace C, Dessole S, Cossu A, Tanda F, Madeddu R, Olivero M, Di Renzo MF. AKT activation drives the nuclear localization of CSE1L and a pro-oncogenic transcriptional activation in ovarian cancer cells. Exp Cell Res 2013; 319: 2627-2636.

25) Hershey CL, Fisher DE. Genomic analysis of the microphthalmia locus and identification of the MITF-J/Mitf-J isoform. Gene 2005; 347: 73-82.

26) Li Y, Yuan S, Liu J, Wang Y, Zhang Y, Chen X, Si W. CSE1L silence inhibits the growth and metastasis in gastric cancer by repressing GPNMB via positively regulating transcription factor MITF. J Cell Physiol 2020; 235: 2071-2079.