Nuclear Export Protein CSE1L Interacts with P65 and Promotes NSCLC Growth via NF- κ B/MAPK Pathway

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Research

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Abstract

Background: Cancer cells can utilize the transportins into and out of the nucleus to stimulate tumor growth and to effectively evade apoptotic mechanisms. We aimed to identify transportins that are overexpressed in Non-small cell lung cancer (NSCLC) and might be targeted to slow the tumor growth.

Methods: Using the ONCOMINE and Gene Expression Omnibus (GEO) databases, we compared the gene expression of 24 importins and exportins in 20 cancer microarray datasets and then analyzed the survival of the candidate genes, CSE1L (Chromosome Segregation Like 1 protein/CAS) was finally selected. The expression level of CSE1L was detected in both NSCLC cells and clinical samples. To evaluate the function of CSE1L *in vitro* and *in vivo*, CCK-8, colony formation, cell apoptosis and subcutaneous tumor model were performed. The co-immunoprecipitation experiment combined with mass spectrometry was conducted to find out the protein interacted with CSE1L. The underlying mechanism was investigated by RNA-seq and Western blot.

Results: 7 transportins were over-expressed in NSCLC patients, and higher expression of CSE1L and XPO5 are associated with shorter survival times. CSE1L was a exportin while the role of tumorigenicity in NSCLC remain unknown. Increased expression of CSE1L in tumor tissues associated with shorter survival times and advanced pathological stage of patients with cancer. The gain- and loss-of-function assays indicated that CSE1L promoted NSCLC cells proliferation and inhibits cells apoptosis. Additionally, we confirmed that CSE1L interacted with RELA (named as P65), and affected its expression in nucleus. RNA-seq results indicated that CSE1L activated the MAPK signaling pathway in NSCLC cells by increasing activity of P65.

Conclusions: We reveal the oncogenic role of CSE1L in NSCLC cancer carcinogenesis. CSE1L promotes proliferation of NSCLC cells by activating the MAPK signaling pathway and inhibiting apoptosis in coordination with P65. Our findings highlight that CSE1L may be a target for the treatment of NSCLC cancer.

Background

According to global cancer statistics, in both sexes combined, lung cancer is the most commonly diagnosed cancer (1-1.6% of the total cases) and the leading cause of cancer death (18.4% of the total cancer deaths), and the leading cause of cancer death among male[1]. Histologically there are 4 major types of lung cancer, including squamous cell carcinoma, adenocarcinoma, large cell carcinoma and small cell carcinoma (SCLC)[2]. The first three types are collectively called non-small cell lung cancer (NSCLC), accounting for approximately 85% of lung cancer case[3]. The 2000s was said to have been an era in which molecular-targeted therapies in NSCLC greatly advanced[4]. Epidermal growth factor receptor (EGFR) gene mutations and Anaplastic lymphoma kinase (ALK) gene rearrangement were discovered[5, 6]. However, non-resectable and recurrent lung adenocarcinoma can only be treated with chemotherapy,

with a poor prognosis and a low survival rate [7], the need to identify more potential therapeutic targets to improve NSCLC treatment is urgent.

Small molecules can passively diffuse through the nuclear pore complex (NPC), whereas larger cargoes, such as mRNA and specific proteins, requires various transport receptors[8, 9]. Nuclear transport receptors (NTRs), called importins, exportins, transportin(s) or, collectively, karyopherins. There are more than 20 NTRs in higher eukaryotes[10, 11]. Export and import of mRNA and specific proteins from the nucleus is a key step in intracellular signaling and can lead to cell proliferation or apoptosis. Cancer cells utilize the processes of nuclear-cytoplasmic transport through the nuclear pore complex (NPC) to stimulate tumor growth and to effectively evade apoptotic mechanisms[12]. NTRs inhibitors are being considered as therapeutic targets against cancer and have shown preclinical anticancer activity[13]. Despite the widespread studies of nuclear import inhibitors, such as INI-43, importazole and ivermectin, none of these agents has been introduced in clinical trials[14]. The development of specific inhibitors of importins is challenging, and many attempts are still in their infancy. By contrast, the development of exportin inhibitors has evolved at a rapid pace. XPO1 (Exportin-1/Chromosome Region Maintenance 1/CRM1) is the main mediator of nuclear export in many cell types[15]. Selinexor, the small- molecule XPO1 inhibitor, was received the FDA approval for the treatment of multiple myeloma (MM) and diffuse large B-cell lymphoma (DLBCL)[16]. Targeting the key mediators of nucleocytoplasmic transport in cancer cells represents a novel strategy in cancer intervention with the potential to significantly affect outcomes.

Here, We use the Oncomine database (<https://www.oncomine.org/resource/login.html>) and Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi>) to screen NSCLC-related NTRs, CSE1L was finally selected. We detected CSE1L expression levels and found that it was upregulated in human NSCLC tissues and correlated with worse outcome. Next, we assessed the effects of CSE1L on cell proliferation, apoptosis, and tumor growth in vivo. Furthermore, we identified that CSE1L interacted with P65 by using Coimmunoprecipitation (Co-IP) and proteomic analyses, verifying the functions and effects of the interacting proteins. Lastly, the MAPK signal pathway responsible for P65 regulation in response to CSE1L-induced tumor progression was also explored. In summary the nuclear exportin CSE1L may be a potential therapeutic target of NSCLC cancer.

Methods

Cell lines and cell culture

The human NSCLC cell lines H1299, PC-9, A549, H1975, H358, H460 and H292 and human embryonic kidney 293T cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). All cell lines were cultured according to ATCC protocols in DMEM or RPMI1640 supplemented with 10% fetal bovine serum (FBS) (Biowest, South America origin), 100 ug/ml penicillin (Sigma-Aldrich, UA), and 100 µg/ml streptomycin (Sigma-Aldrich, UA) at 37°C in a humidified incubator under 5% carbon dioxide. These cell lines were mycoplasma-free and authenticated by quality examinations of morphology and growth profile.

Database analysis

We performed the ONCOMINE database combined with GEO database to screen NSCLC-associated NTRs. The expression data for 24 NTRs[11] were available from ONCOMINE. GEO datasets could be downloaded from NCBI. The survivals for the screened genes were analyzed in 117 lung adenocarcinoma patients (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE13213>) and 293 lung tumor samples (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE30219>). 503 lung adenocarcinoma patients from TCGA cohort (<https://cancergenome.nih.gov/>) was used to analyze the correlation between CSE1L mRNA levels and NSCLC clinicopathological features.

Human clinical specimens

Fresh human non-small cell lung cancer tissues and matched adjacent noncancerous tissues for real-time PCR and Western blot analyses were collected from the Department of Cancer at Huashan Hospital affiliated with Fudan University, Shanghai, China between 2011 and 2018. During the operation, human surgical specimens were immediately frozen in liquid nitrogen and stored at -80°C for further investigation. All of the tissue specimens for this study were obtained with patient informed consent. The study was approved by the Ethics Committee of the Ethics Committee of Fudan University.

Tissue microarray

Two tissue microarrays containing 75 and 25 paired NSCLC tissues and matched adjacent noncancerous tissues was purchased from Shanghai Biochip Co. Ltd. (Shanghai, China). Immunohistochemical staining was performed to detect the protein expression level of CSE1L in NSCLC tissues and matched noncancerous tissues. The average gray value of the image was used as a quantitative evaluation of the expression level using Image-Pro Plus 6.0 software. The proportion of the stained cells and the extent of the staining were used as the criteria for evaluation. The percentage of positive cells was scored as: < 5% (0), 5%-25% (1), 25%-50% (2), 50%-75% (3), and > 75% (4). The intensity of staining was scored as: no staining (0), light brown (1), brown (2), and dark brown (3). The final IHC scores was obtained using the traditional scoring method. IHC scores were calculated as the product of intensity (0 to 3) and the percentage of positive cells (0 to 4), yielding a range from 0 to 12.

RNA extraction and real-time polymerase chain reaction assay

Total RNA was extracted from the lung cancer cells and tissues using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. cDNA was synthesized by random primers and the PrimeScript RT Reagent Kit (Takara, Dalian, China). The primer sequences for real-time PCR are shown in Supplementary Table 1. Real-time PCR was performed using SYBR Premix Ex Tag (Takara, Dalian, China). The PCR conditions were as follows: 95°C for 15 s followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. β -actin was used as the internal control.

Vector construction and RNA interference

The coding sequences (CDS) of human CSE1L was commercially synthesized (GENEWIZ), then was cloned and inserted into the lentiviral expression vector pWPXL. shRNA targeting CSE1L as well as a negative control (shNC) were obtained from GeneChem (Shanghai, China). To produce lentivirus containing CSE1L, 293T cells were cotransfected with the pWPXL- CSE1L and the lentiviral vector packaging system using Lipoectamine 2000.

Small-interfering RNA (siRNA) oligonucleotides for CSE1L and P65 were designed and synthesized by RiboBio (Guangzhou, China). The sequences for the siRNAs are shown in Supplementary Table 2. Transient transfection was performed using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. After transfection for 48 h, the cells were used for functional assays, including apoptosis, migration, invasion, colony formation, RNA extraction, and Western blot.

Protein extraction and Western blotting

Cell and tissue proteins were extracted using T-PER® Protein Extraction Reagent (Thermo Scientific, USA) with a phosphatase inhibitor cocktail (Roche Applied Science, Switzerland) and a proteinase inhibitor cocktail (Roche Applied Science, Switzerland). We used the Minute™ Cytoplasmic and Nuclear Extraction Kit (Invent Biotechnologies, USA) to separate the nuclear and cytoplasmic. The protein concentrations were determined using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, USA). Proteins were separated by SDS-PAGE gels and transferred to nitrocellulose (NC) filter membranes (Millipore, Massachusetts, USA) or polyvinylidene fluoride (PVDF) membranes (Millipore, Massachusetts, USA). The membranes were incubated with primary antibodies overnight at 4°C and probed with secondary antibodies at room temperature for 1~2 h. The antibodies used were shown in Supplementary Table 3.

Cell proliferation and colony formation assay

The relative cell proliferation was monitored using CCK-8 (Dojindo, Japan) according to the manufacturer's protocol. Briefly, cells were seeded in 96-well plates at 1×10^3 cells per well. According to the manufacturer's instructions, 10 μ l of CCK-8 solution and 100 μ l medium was added to each well, and the mixture was incubated at 37°C for 2 h. The absorbance was measured at 450 nm.

For the colony formation assay, a total of 0.5×10^3 cells were seeded in 6-well plates and cultured at 37°C in a humidified incubator at 5% carbon dioxide. Two weeks later, the cell colonies were washed with PBS, fixed with methanol, and stained with 0.1% crystal violet (1 mg/mL) for 30 minutes. All of the experiments were repeated in triplicate and assessed under a light microscope.

Flow cytometry for cell apoptosis and cell cycle analysis

The human NSCLC cell lines (PC-9, H1299) stably interference CSE1L or the lentiviral vector or transfected with p65 si-RNAs or si-NC, A549 cells and H292 cells stably expressing CSE1L or the lentiviral vector and transfected with p65 si-RNAs or si-NC were used for the experiments. A flow cytometer (BD LSR II, BD Biosciences, USA) was used to detect the cell phenotype. Apoptosis was measured via the

Apoptosis Detection Kit (BD Biosciences, USA) according to the manufacturer's protocol. The data was analyzed using FlowJo software version 8.6.3 (FlowJo, USA). Cell cycle distribution was measured by the PI Cell Cycle Detection Kit (Beyotime Biotechnology, China) according to the manufacturer's protocol. The results were analyzed using ModFit software (BD Biosciences, USA).

Cell migration and invasion assays

Cell migration and invasion assays were performed by Transwell filter chambers (BD Biosciences, New Jersey, USA). For migration assays, 5×10^4 cells in 200- μ L of serum-free culture medium were suspended into the upper chamber with the noncoated membrane. For invasion assays, 1×10^5 cells in 200- μ L of serum-free culture medium were placed into the upper chamber with the Matrigel-coated membrane diluted with serum-free culture medium. An 800- μ L culture medium supplemented with 10% FBS was added in the lower chamber. After incubation at 37 °C in a humidified incubator under 5% carbon dioxide, the cells in the bottom surface of the membrane were fixed with 100% methanol, stained with 0.1% crystal violet for 30 min, and counted under a light microscope (Olympus, Japan).

Immunofluorescence

Cells were plated in six-well plates on glass coverslips. The cells were fixed with 4% formaldehyde for 15 minutes at 4°C, then infiltrated with 0.3% Triton X-100 for 15 minutes. After washing three times with PBS, the wells were treated with blocking solution for 30 minutes and then incubated overnight with anti-CSE1L (abcam, 1:50) and anti-P65(CST, 1:50) antibody at 4°C. After incubation with the secondary antibody (anti-rabbit (invitrogen, 1:300), anti-mouse (invitrogen, 1:150)) at room temperature for 1 hour, DAPI was used to stain the nucleus. A confocal laser scanning microscope (Leica TCS-SP5, Leica Microsystems, Germany) was used to observe the images.

Co-immunoprecipitation and mass spectrometry

When the cells fullness reached more than 90%, the cells were scraped off directly with a cell scraper using ice-cold RIPA lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) with protease and protein phosphatase inhibitors (Roche Applied Science, Switzerland). Three milligrams of protein were pre-cleared with 30 μ L of protein A/G magnetic beads (Millipore, Massachusetts, USA) at 4°C for 2 h. The beads were removed, and 5 μ L of the primary antibody (CSE1L or P65) or isotype IgG was added to the supernatant at 4°C overnight with gentle mixing on a rocking platform to capture the fusion proteins. Then, 40 μ L of protein A/G magnetic beads was added to each immunoprecipitation mixture for 4 h at 4°C. The magnetic beads were collected by placing the tube in the appropriate magnetic separator. The beads were washed three times with PBS/0.1% Triton. The magnetic beads isolated using a magnetic rack and then boiled in 2 $\times$ sodium dodecyl sulfate (SDS) loading buffer. The bound fusion proteins were separated by SDS-PAGE and stained with a Silver Staining Kit (Beyotime Biotechnology, Shanghai, China). The proteins in the SDS-PAGE were digested with trypsin, and then analyzed by Triple TOF 5600 mass spectrometer (AB Sciex, Texas, USA). Protein identification was performed using Protein Pilot 4.5 (AB Sciex, Texas, USA).

In vivo tumor growth

All animal experiments were approved by the Animal Ethics Committee of the Shanghai Cancer Institute. Six- to eight-week-old female BALB/c-nu/nu mice were bred by Shanghai Cancer Institute (Shanghai, China) and housed in [specific pathogen-free \(SPF\) conditions in a laboratory animal](#) facility.

For the *in vivo* xenograft assays, 3×10^6 A549 cells and H292 cells stably expressing CSE1L or the lentiviral vector and 2×10^6 PC-9 cells and H1299 cells stably expressing shCSE1L or the negative control were separately subcutaneously inoculated into the dorsal right flanks of the nude mice (6 or 8 per group). The tumor size was measured two times every week. The tumor volume (V) was measured by calipers and calculated according to the following formula: $(\text{length} \times \text{width} \times \text{width})/2$. After eight or ten weeks, the mice were sacrificed, and the tumors were weighing.

Statistical analysis

Differences among variables were assessed by χ^2 analysis or two-tailed Student's t-tests. Difference in survival were analyzed using the log-rank test. The correlation of CSE1L and P65 expression was examined by Spearman's correlation test. Data are presented as the mean $\pm$ standard deviation (SD) or the mean $\pm$ standard error of the mean (SEM). Differences were considered statistically significant at $P < 0.05$.

Results

CSE1L was upregulated in NSCLC and associated with poor outcomes

We screened NSCLC-related NTRs by utilizing the ONCOMINE database and GEO database. First, we analyzed the mRNA expression of 24 NTRs in lung cancer from ONCOMINE database. 7 NTRs (KPNA2, TNPO1, IPO13, XPO1, CSE1L, XPO5, XPOT) exerted higher mRNA expression in patients with lung cancer, while 17 NTRs (KPNA1, KPNA3, KPNA4, KPNA5, KPNA6, KPNB1, TNPO2, TNPO3, IPO4, IPO5, IPO7, IPO8, IPO9, IPO11, XPO4, XPO6, XPO7) had no change compared with the normal lung tissues (Figure S1A). Next, we analyzed survival of the alternative 7 nuclear transporters in GEO database (GSE30219 and GSE13213). Survival analysis showed that higher expression level of CSE1L and XPO5 were significantly associated with worse outcome of lung cancer patients in the two databases (Figure S1B, C and Fig. 1A, B), while the survival of higher expression level of KPNA2, TNPO1, XPO1 and XPOT in GSE30219 cohort and IPO13 in GSE13213 cohort were no significant difference. Taken together, the results revealed that higher expression of CSE1L and XPO5 closely related to the develop of lung cancer. XPO5 has been reported to promote lung cancer cell proliferation and apoptosis[17, 18], while the role of CSE1L in NSCLC remains unknown, Therefore, CSE1L was selected as the research target.

To further assess the clinical significance of CSE1L, 503 lung adenocarcinoma patients TCGA cohort was used to analyze the correlation between CSE1L mRNA expression levels and NSCLC clinicopathological features. CSE1L was highly expressed in NSCLC tumor tissues compared with normal tissues. Importantly, high level of CSE1L was positively correlated with advanced pathological TNM stages (Fig. 1C). Next, we detected CSE1L protein expression level by IHC staining assay in the NSCLC tissue microarray which contained 100 pairs of NSCLC tissues and matched normal tissues. The representative IHC staining results are shown in Fig. 1D. The IHC results showed that CSE1L was significantly overexpressed in NSCLC tissues compared to the matched normal tissues. The clinical analysis showed that the protein expression level of CSE1L had no significant correlated with gender, age, histological grade, clinical stage and T/N stage (Supplementary Table 4). Next, we analyzed CSE1L mRNA expression level in another 67 paired human NSCLC and their corresponding noncancerous lung tissues by real-time PCR. Compared with corresponding nontumorous tissues, CSE1L was significantly upregulated in NSCLC tissues. Moreover, the mRNA expression level of CSE1L was upregulated in 80.6% (54/67) of NSCLC cases (Fig. 1E). The mRNA expression level of CSE1L had no significant correlated with clinicopathologic characteristics (Supplementary Table 5). To investigate the functional roles of CSE1L in NSCLC cells, the mRNA and protein levels of CSE1L were detected in NSCLC cell lines (Figure S2A and B). Taken together, our data showed that CSE1L was highly expressed in NSCLC and associated with poor outcomes.

CSE1L exerted a tumor promoting effect on NSCLC both *in vitro* and *in vivo*

First, we interfered the expression of CSE1L by using si-RNAs (Fig. 2A), and established stable models of CSE1L overexpressed in A549 and H292 cell lines (Fig. 3A). CCK-8 assays indicated that CSE1L significantly inhibited the proliferation abilities of NSCLC cells compared with control cells (Fig. 2B), whereas overexpression of CSE1L substantially promoted the proliferation abilities compared with control cells (Fig. 3B). Cell cycle analysis demonstrated that the percentage of cells in G2/M phase was increased in H1299 cells and the percentage of cells in S phase was reduced in PC-9 cells after CSE1L knockdown (Fig. 2C and D). Whereas the percentage of cells in G0/G1 and S phase were enhanced and G2/M was reduced when overexpressed CSE1L in A549 cells (Fig. 3C). Moreover, the migration and invasion assays showed that the knockdown of CSE1L in PC-9 and H1299 cells significantly inhibited the cell migration and invasion potential ($P < 0.05$, Figure S3A and B). Then we established stable models of CSE1L knockdown in H1299 and PC-9 cell lines (Figure S2C and D). Colony formation assays showed that knockdown of CSE1L inhibited cells growth, whereas overexpression of CSE1L got the opposite result (Fig. 2E and 3D). Moreover, CSE1L knockdown promoted apoptosis whereas overexpression of CSE1L significantly suppressed apoptosis in NSCLC cells (Fig. 2F and 3E). To evaluate the effect of CSE1L *in vivo*, mouse xenograft models were used. The results showed that tumor growth rate was lower in sh-CSE1L groups with smaller tumor volumes and lower tumor weights than negative control group ($P < 0.05$) (Fig. 2G, H). In contrast, stable overexpression of CSE1L significantly increased the tumor volumes and tumor weights than control groups ($P < 0.05$) (Fig. 3F and G). There was no significant difference between the four groups in terms of body weight (Figure S4). Furthermore, we detected the change of protein levels of apoptosis-related markers after CSE1L knockdown. Down-regulation of CSE1L suppressed the expression of p-BCL-2, BCL-XL, BCL-W, BECN1, and MCL-1, and promote the expression of

BAX, BAK and p-BAD (Fig. 2E). In conclusion, these results indicated that CSE1L acted as an important tumor promoting factor in NSCLC cells both *in vitro* and *in vivo*.

Cse1l Interacted With P65 And Stabilizes P65 In Nucleus

A vast amount of studies supporting the concept that CSE1L is a pro-tumor gene, but the role of CSE1L in NSCLC has not been explored clearly. In this study, Co-IP combine with MS analysis were used to screen the potential interacted protein of CSE1L to investigate the further mechanism. IP samples of CSE1L extracted from H1299 and PC-9 cells were examined in SDS/PAGE followed by silver staining, and the location of CSE1L was indicated (Figure S4A). Next, the IP samples were subjected to in-gel trypsin digestion and extracted to MS analysis. Through MS analysis, eight proteins (UBC, P65, RAN, KPNB1, MSH6, SSBP1, CTPS2 and ATP50) that may interact with CSE1L were identified both in H1299 and PC-9 (Figure S4B). As CSE1L was identified as a nucleoprotein in the Uniprot, we priorly select the proteins that located in nucleus. Interestingly, the protein MSH6, which we had verified previously that could interacted with CSE1L[19], was identified in the list, so we choose the remaining four nuclear proteins for further study. Co-IP-WB was employed to confirm whether these four proteins were interacted with CSE1L. The results confirmed that RAN, UBC and KPNB1 were not interacted with CSE1L (Figure S4C), but P65 was interacted with CSE1L in H1299, PC-9 and 293T cells (Fig. 4A). Meanwhile, CSE1L was also determined from immunoprecipitation isolated by P65 antibody in H1299, PC-9 and 293T cells (Fig. 4A). Importantly, an immunofluorescence assay showed co-localization of CSE1L and P65 in NSCLC cells (Fig. 4B). These evidences collectively suggested that there was an endogenous interaction between CSE1L and P65. To further study the mechanism of the interaction between CSE1L and P65, we interfered the expression of CSE1L and P65, respectively. WB showed a significant decrease in P65 protein expression when CSE1L was down-regulated. In contrast, P65 was increased when CSE1L was up-regulated (Fig. 4C). However, at the mRNA level, there were no significant changes in the expression of P65 after CSE1L knockdown or overexpression (Figure S4D). Then we performed nuclear and cytoplasmic separation test, we found that P65 was reduced in the nucleus in CSE1L knockdown cells while CSE1L had no changed in P65 knockdown cells (Fig. 4D and E). The immunofluorescence assay also showed the same results (Fig. 4F). These datats indicated that P65, as a downstream protein, was regulated by CSE1L in nucleus rather than cytoplasm. After cycloheximide (CHX) blocked protein synthesis, the half-life of P65 protein in CSE1L knockdown cells was significantly shorter than the control cells, in contrast, the half-life of P65 protein in CSE1L overexpressed cells was longer that the control cells (Fig. 4G and H). Taken together, these evidences demonstrated that CSE1L interacted with P65 in nucleus in NSCLC cells and promoted the stabilization of P65.

CSE1L promoted cell proliferation and inhibited apoptosis partly through P65

Our findings demonstrated that CSE1L could promote cell proliferation and inhibit cell apoptosis. However, whether CSE1L functions through P65 is largely unknown. First, P65 specific siRNA was used to knockdown in H1299 and PC-9 cells (Fig. 5A), then the CCK-8 and apoptosis assays were carried on. The

results demonstrated that P65 had the same function with CSE1L in NSCLC cells (Figure. 5B and C). Since CSE1L interacts with P65 and affects its protein expression and stability in NSCLC cells, it is plausible that CSE1L functions through P65 and that P65 has an important role in NSCLC cell. To test this hypothesis, P65 was knockdown in CSE1L overexpressing A549 and H292 cells (Fig. 5D and E). Then, colony formation and apoptosis assays were used to measure cell growth. We found that P65 knockdown significantly inhibited the growth of the CSE1L-overexpression induced cell growth (Figure. 5F and G), and significantly promoted the apoptosis of the CSE1L-overexpression induced cell apoptosis (Fig. 5H and I). In summary, these evidences revealed that CSE1L promoted cell proliferation and inhibited apoptosis possibly through P65.

CSE1L regulated P65 and promoted NSCLC cell proliferation and inhibited apoptosis by activating the NF- κ B /MAPK signaling pathway

Currently, the identities of CSE1L-associated signaling molecules that are responsible for mediating human lung cancer cell proliferation and apoptosis are unclear. To further elucidate the molecular mechanism of CSE1L in NSCLC, RNA-seq was utilized to elucidate the total transcriptional changes of H1299 cells after the down-regulation of CSE1L expression. The KEGG pathway analysis for the result of RNA-seq indicated that CSE1L affected many pathways, such as MAPK signaling pathway, p53 signaling pathway, apoptosis, ferroptosis and TNF signaling pathway (Fig. 6A). Meanwhile, gene set enrichment analysis (GSEA) was utilized to reveal the gene signature affected by CSE1L down-regulation. The process of apoptosis and TNF signaling pathway were enriched by GSEA (Fig. 6B, C). The results of qRT-PCR experiments confirmed that the apoptosis process and TNF signaling pathway were significantly affected after interfering with the expression of CSE1L (Fig. 6D, E). As mentioned above, these results also supported the standpoint that the apoptosis of the NSCLC cells was affected by the expression of CSE1L. Moreover, we had proved that P65 interacted with CSE1L, and the P65 was a key protein in the MAPK signaling pathway and TNF signaling pathway (Fig. 6F). To further verify these results, WB was used to detect the alteration of expression for key node proteins in the MAPK signaling pathway after CSE1L knockdown in NCI-H1299 cells and CSE1L overexpressed in A549 cells. The results showed that the apoptosis key molecules of p-JNK, ITCH, CREB, caspase 8, caspase 3 and caspase 7 were changed obviously, and the MKK7 and total of JNK protein levels were not significantly altered (Fig. 6G). Meanwhile, the DNA key proteins of p-MEK1/2, p-ERK1/2 and p-MSK1 were significantly altered, and the total of MEK1/2, ERK1/2 and MSK1 proteins had no change (Fig. 6H). The results indicated that CSE1L could regulate the protein expression level of P65, and promoting the cell proliferation and apoptosis through MAPK signaling pathway.

High expression of CSE1L and P65 was significantly associated with worse prognosis in NSCLC patients

To determine whether the expression of CSE1L and P65 in NSCLC were related to the prognosis of patients with lung cancer, we performed western blotting analysis in 40 pairs of cancerous and noncancerous fresh tissues from NSCLC patients. The expression levels of CSE1L and P65 in the 40 NSCLC tissues were upregulated than those in adjacent noncancerous tissues (Fig. 7A). In 40 NSCLC

specimens, high expression of CSE1L was found in 33 cases of NSCLC (82.5%), and high expression of P65 was found in 35 cases of NSCLC (87.5%) (Fig. 7B). Importantly, CSE1L protein expression was positively correlated with P65, suggesting a potential CSE1L-P65 pathway in lung cancer tissues ($R = 0.7794$, $P < 0.0001$) (Fig. 7C). The protein expression level of CSE1L and P65 were detected in the 7 NSCLC cell lines, and had the similar results ($R = 0.9262$, $P = 0.0027$) (Fig. 7D). Moreover, in 25 lung adenocarcinoma tissue microarray, CSE1L protein expression was positively correlated with P65 ($R = 0.4699$, $P = 0.0178$) (Fig. 7E). To further explore the role of P65 in predicting cancer prognosis, we analyzed the P65 expression from the publicly available GEO database (GSE13213, $n = 117$). The survival analysis showed that higher expression levels of P65 were strongly associated with shorter survival time for lung cancer patients (Fig. 7F). The ROC curves illustrated that the areas under the curve of the CSE1L- and P65-based predictions were 0.660 and 0.612, respectively, suggesting that they could both potentially be applied for the prediction of patient survival (Figure S5). To further explore the role of CSE1L and P65 in predicting cancer prognosis, we analyzed the CSE1L and P65 mRNA expression and the corresponding clinical data from the publicly available GEO database (GSE13212, $n = 117$). Interestingly, the shortest survival time was observed in the group with the highest expression of both CSE1L and P65 (Fig. 7G), and the ROC curve of CSE1L and P65 was 0.674 (Figure S5D). Taken together, these findings indicate that CSE1L-P65 might be potential prognostic biomarkers for this disease.

Discussion

CSE1L, a key player in the nuclear transport pathway, is the human homologue of the yeast gene CSE1 that contains a 971-aa open reading frame, and it encodes a protein of about 100 KDa in molecular mass that distributes in the nuclei and cytoplasm of cells[20]. The DNA fragment was first isolated by Brinkmann et al in 1995 in MCF-7 breast cancer cells[21]. CSE1L was found as a multiple functional protein that plays roles in apoptosis, cell proliferation and survival[22], microvesicle formation[23], nucleocytoplasmic transport[24], and cancer metastasis[25, 26]. The CSE1L gene maps to 20q13, a locus often amplified in cancers of various origin, might lead to genetic instability, and is associated with chromatin and regulates expression of select p53 target genes[27]. CSE1L is highly expressed in tissues that have a high mitotic index such as human tumor cells[28], fetus liver and testes, and CSE1L expression is correlated with cancer progression and metastasis in some cancer types[26, 29]. Thus, CSE1L may have clinical applications in the diagnosis, therapy, and prognosis of cancer.

Tumor suppressor protein and cell cycle regulatory protein may be abnormal after the process of nuclear-cytoplasmic transport, and cell apoptosis may be inactivated, which is essential for tumor growth and development[30]. Dysregulated CSE1L expression and localization has been reported to correlate with cancer progression, and its abnormal distribution has been proposed to be a biomarker for prognosis of carcinomas[31]. CSE1L could interact with mutS homolog 6 (MSH6) and positively regulate the MSH6 protein to promote osteosarcoma progression in our previous study[19]. However, the clinical significance and the biological functions and molecular mechanisms of CSE1L in lung cancer remain elusive. In the present study, we have demonstrated that higher expression of CSE1L was observed in NSCLC tissues compared to that in adjacent noncancerous tissues. Increased CSE1L expression level was significantly

associated with advanced tumor stages as well as adverse patient outcome. It has been previously reported that CSE1L showed distinct localization patterns in different cell lines. CSE1L was consistently accumulated in the nucleus of ovarian cancer cells, while it was mainly localized in the cytoplasm of the MCF7 breast carcinoma cells and was uniquely cytoplasmatic in the HT-29 colon carcinoma cells[32, 33]. CSE1L was also a secretory protein which existed in the body fluids of human especially in the blood of cancer patients[34]. While there is few studies of CSE1L in lung cancer. The observations that CSE1L was predominantly localized in the nucleus and cytoplasm. It was interesting that CSE1L was also observed in membrane in NSCLC cells. In line with its different localization, CSE1L might play different roles. These findings indicated that CSE1L can be used as a clinical biomarker of prognosis in NSCLC, and it plays an important role in the development and progression of lung cancer.

CSE1L was first cloned while searching for genes that rendered breast cancer cells resistant towards toxin induced[21]. CSE1L is essential for cell survival and cell apoptosis. There were studies reported that CSE1L knockdown inhibited the proliferation of cancer cells and also increased cell apoptosis in colorectal cancer cells and gastric cancer cells[26, 35]. In our study, a series of *in vitro* and *in vivo* assays were conducted to clarify the biological functions of CSE1L in regulating NSCLC cells. We found that CSE1L could increase the proliferation and protect apoptosis of lung cancer cells *in vitro* and promoted the growth of lung cancer cells *in vivo*. These findings match with the knowledge that CSE1L is essential for cancer cell growth and development, and we can confirm that CSE1L promotes cancer progression via the stimulation of cancer cell proliferation as well decrease cells apoptosis. CSE1L functions in mitotic spindle checkpoint, which is very important for cell cycle[28]. Herein, it was interested that we found that down-regulation of CSE1L had different effect in NSCLC cells. CSE1L knockdown arrested the cell cycle progression in the G2/M phase in H1299 cells and reduced the percentage of PC-9 cells in S phase, and CSE1L overexpressssion had the opposite results both in G2/M phase and S phase. Both of the results indicated that CSE1L promoted NSCLC cells growth. All of these evidences indicated that CSE1L was crucial to the pathogenesis of NSCLC and may be a new therapeutic target for patients with NSCLC.

To date, the molecular mechanisms by which CSE1L promoting lung cancer cell proliferation and inducing apoptosis have not been elucidated. Our datats demonstrated that CSE1L could specifically interact with P65 to form a complex in the nuclei of NSCLC cells. The transcription factor nuclear factor- κ B (NF- κ B) regulates the expression of a wide variety of genes involved in immune and inflammatory responses, cell proliferation, tumorigenesis, cell survival, and development[36]. The P65 plays a central role in the NF- κ B pathway [37]. The protein level of P65 was notably altered in the nucleus when CSE1L was knockdown or overexpressed, and CSE1L was important for stabilization of P65 by protecting it from proteasomal degradation. We also showed that knockdown of P65 significantly inhibited the proliferation and induce apoptosis of NSCLC cells *in vitro*, suggesting that P65 has an indispensable role in lung cancer. P65 knockdown reduced the enhancement of CSE1L on NSCLC cells proliferation and promoted apoptosis. Survival analysis showed that high expression of P65 was significantly associated with a short survival time in lung cancer patients. Moreover, the shortest survival time was observed in the group with high expression of both CSE1L and P65. These findings further confirmed the correlation between

CSE1L and P65 and indicated that the interaction between CSE1L and P65 was responsible for the oncogenic function of CSE1L.

As of today, the regulatory mechanism of the CSE1L signaling pathway on NSCLC cancer progression is still obscure; Only a few studies have reported on the interaction of CSE1L with other cancer signaling pathways. A study suggested that AKT activation forces the nuclear accumulation of CSE1L in the ovarian cancer cell, likely to induce prooncogenic signals[33]. Another study revealed that CSE1L inhibition decreased MITF and suppressed GPNMB expression, thereby activating the PI3K/Akt/mTOR and MEK/ERK signaling pathway, ultimately inhibiting the tumor growth and metastasis in gastric cancer[26]. In melanogenesis of melanoma cells, CSE1L links and controls cAMP/PKA and Ras/ERK signal pathways, and may be a potential target for treating melanomas[38]. We know that there are two distinct NF- κ B signaling pathways: 1) the classical pathway, primarily activated by pathogens and inflammatory mediators, and 2) the alternative pathway, which involves the NF- κ B-inducing kinase (NIK, also known as MAP3K14)[37]. P65 plays a key role in the regulation of MAPK signal pathway[39, 40]. It was reported that while ERK was localized in the cytoplasm of resting cells, many of its substrates are nuclear, and indeed, extracellular stimulation induces a rapid and robust nuclear translocation of ERK[41]. In our study, CSE1L activated ERK phosphorylation by effecting the protein expression of P65 possibly in nuclear, then promoted NSCLC cell proliferation and regulated cell cycle. JNK is involved in cancer cell apoptosis, proliferation, autophagy and tumor immune evasion[42]. Here, overexpressed of CSE1L activated JNK signal axis, thereby affecting the apoptotic signal. Consistently, We hypothesize that in the nucleus, CSE1L binds to the nuclear export signal (NES) on P65 and RAN in its active GTP-bound form (RAN-GTP). The complex is subsequently docked to NPC and passes through the NPC into the cytoplasm. Hydrolysis of RAN-GTP to RAN-GDP causes the disassembly of CSE1L-P65 complex and release of P65 and CSE1L in the cytoplasm, then activate the NF- κ B/MAPK pathway, resulting in the promotion of cell proliferation and the inhibition of cell apoptosis (Fig. 8).

Conclusions

In summary, our findings demonstrate that CSE1L and P65 play important roles in the development and progression of NSCLC. Meanwhile, CSE1L could interact with and mediate the expression of downstream genes P65, then activates the NF- κ B/MAPK pathway, resulting in the promotion of NSCLC cell proliferation and the inhibition of cell apoptosis. More importantly, our study suggested that CSE1L may serve as a promising therapeutic target for the prevention and treatment of NSCLC.

Abbreviations

MAPK signaling pathway: The mitogen-activated protein kinase signaling pathway

NCBI: National Center for Biotechnology Information

PBS: Phosphate buffer saline

PI: Propidium

DMEM: Dulbecco's Modified Eagle's Medium

RPMI 1640: Roswell Park Memorial Institute 1640

PCR: Polymerase chain reaction

IHC: Immunohistochemistry

CCK-8: Cell Counting Kit-8

DAPI: 4',6-Diamidino-2-Phenylindole

RIPA: Radio Immunoprecipitation Assay

TNM: Tumor Node Metastasis

ROC: Receiver operating characteristic

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Shanghai Jiao-tong University and the Ethics Committee of Fudan University (Shanghai, China).

Consent for publication

All authors agree on publication of the results of the present manuscript.

Availability of data and materials

All data generated or analyzed during this study are included either in this article or in the supplementary information files.

Competing interests

The authors declare that they have no competing interest.

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Authors' contributions

Hechun Lin, Ming Yao and Hong Li were in responsible for the design of the study and wrote the manuscript; Dongdong Chen participated in the mass spectrometry analysis and bioinformatic analysis; Hechun Lin and Jing Li culture the cells and conduct the stable cell lines; Tao Yu performed qPCR, Western blot validation and the study of the signaling pathway; Q Geng helped conduct the migration and invasion assay; MiaoXin Zhu was in charge of the animal imaging detection. FY Zhao and HW Kong helped some animal experiments; All of the authors reviewed the manuscript before submission and approved the final manuscript.

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Supplemental Information

Figure S1. Screening NSCLC-related nuclear and cytoplasmic importins and exportins. (A) The mRNA expression of 17 importins and 7 exportins in different cancer types by using the ONCOMINE database. (B) The survivals of the 6 nuclear transporters in GEO database GSE30219. (C) The survivals of the 6 nuclear transporters in GEO database GSE13213.

Figure S2. The expression level of CSE1L (A) The mRNA and protein expression level of CSE1L in human NSCLC cell lines were measured by Western blotting and real-time PCR (B). (C) The expression levels of CSE1L were determined by Western blotting analyses and real-time PCR (D) after sh-CSE1L or sh-NC in H1299 and PC-9 cells.

Figure S3. Knockdown of CSE1L inhibited NSCLC cell migration and invasion. (A) The effect of knockdown CSE1L was assessed by migration and invasion assays in H1299 and PC-9 cells (B).

Figure S4. Weights of the xenograft tumor model mice on indicated days. (A) PC-9 cells. (B) H1299 cells. (C) A549 cells. (D) H292 cells. Values represent the mean $\pm$ SD.

Figure S5. Co-IP-MS was performed to screen the interacting proteins of CSE1L. (A) Co-IP assay was employed to determine the protein interaction with CSE1L. Immunoprecipitate isolated by CSE1L antibody was detected in SDS/PAGE and followed by silver staining. (B) The potential interacting proteins of CSE1L identified both in H1299 and PC-9 cells. (C) The expression of P65 in CSE1L knockdown cells and overexpression cells.

Figure S6. P65 and CSE1L were correlated with the poor prognosis of NSCLC patients. (A) The receiver operating characteristic (ROC) curves for predicting patient survival time using CSE1L expression. (B) The receiver operating characteristic (ROC) curves for predicting patient survival time using P65 expression. (C) The receiver operating characteristic (ROC) curves for predicting patient survival time using CSE1L and P65 expression.

Supplementary Table 1. The primers used in this study.

Supplementary Table 2. The sequences for the siRNAs used in this study.

Supplementary Table 3. The primary antibodies used in this study.

Supplementary Table 4. Correlation between CSE1L mRNA expression levels in NSCLC patients and their clinicopathologic characteristics.

Supplementary Table 5. Correlation between tissue CSE1L protein expression levels in NSCLC patients and their clinicopathologic characteristics.

Supplementary Table 6. Correlation between tissue P65 protein levels in NSCLC patients and their clinicopathologic characteristics.

Figures

Figure 1

CSE1L is upregulated in NSCLC and correlated with the poor outcome. (A) The survivals of the CSE1L in GEO database GSE30219. (B) The survivals of CSE1L in GEO database GSE13213. (C) The expression of CSE1L in 503 lung adenocarcinoma patients TCGA cohort and the correlation between CSE1L mRNA levels and NSCLC patients TNM stages. (D) The protein expression level of CSE1L in human NSCLC tissues microarray was measured by IHC. The representative of IHC staining images of tumor and adjacent tissue are shown in the left and the statistics scores is in the right. (E) The mRNA expression level of CSE1L in human NSCLC tissues and noncancerous was measured by real-time PCR. Error bars represent the SEM and SD. *P < 0.05; **P < 0.01.

Figure 2

Effects of knockdown of CSE1L on NSCLC cell proliferation and apoptosis. (A) The protein expression levels of CSE1L in CSE1L knockdown and negative control H1299 and PC-9 cells were determined by Western blotting analyse (B) The effect of si-CSE1L on cell proliferation was evaluated by a CCK-8 assay in H1299 and PC-9 cells. (C) Flow cytometry was employed to detect the cell cycle after si-CSE1Ls in H1299 cells and PC-9 cells (D). (E) The effect of knockdown CSE1L on cell proliferation was evaluated by

a colony formation assay in H1299 and PC-9 cells. (F) Flow cytometry was employed to detect the apoptosis percentages of the CSE1L knockdown cells and control cells. (G) and (H) The xenograft tumors formed by the sh-CSE1L targeted and negative control H1299 and PC-9 cells. The weight of the xenograft tumors and the growth curve demonstrating the tumor volumes on indicated days were shown in the middle and the right. (I) WB was used to detect the apoptotic-related markers in CSE1L knockdown H1299 and PC-9 cells. Error bars represent the SEM and SD. *P < 0.05; **P < 0.01.

Figure 3

Effects of overexpress of CSE1L on NSCLC cell proliferation and apoptosis. (A) The mRNA and protein levels of CSE1L in CSE1L overexpression and negative control A549 and H292 cells were determined by real-time PCR and Western blotting analyses, respectively. (B) The effect of CSE1L on cell proliferation was evaluated by a CCK-8 assay in A549 and H292 cells. (C) Flow cytometry was employed to detect the cell cycle of the CSE1L overexpression cells and control cells. (D) The effect of CSE1L on cell proliferation was evaluated by a colony formation assay in A549 and H292 cells. (E) Flow cytometry was employed to detect the apoptosis percentages of the CSE1L overexpression cells and control cells. (F) and (G) The xenograft tumors formed by the sh-CSE1L targeted and negative control A549 and H292 cells. The weight of the xenograft tumors and the growth curve demonstrating the tumor volumes on indicated days were shown in the middle and the right. Error bars represent the SEM and SD. *P < 0.05; **P < 0.01.

Figure 4

CSE1L interacted with P65 and stabilized the P65 protein expression in NSCLC cells. (A) Co-IP was carried out using an anti-CSE1L antibody (upper diagram) or anti-P65 antibody (lower diagram). The immunoblotting assay detected anti-CSE1L and anti-P65 antibodies, respectively. IgG was used as a negative control. (B) Immunofluorescence staining was used to detect the colocalization and coexpression of the CSE1L and P65 proteins in the nucleus. (C) The regulatory relationship between

CSE1L and P65 was verified by Western blotting. (D) and (E) Nuclear and cytoplasmic separation test was carried on to measure the expression of CSE1L and p65 in nucleus and cytoplasm when si-CSE1L or si-P65. β -actin and lamin A/C were used as an internal control (F) Immunofluorescence staining was used to detect the colocalization when si-CSE1L or si-P65. (G) PC-9 cells with CSE1L knockdown and A549 cells overexpressing CSE1L were treated with CHX (100 μ g/ml) for the indicated time points. The cell lysates were examined by Western blot.

Figure 5

CSE1L promoted cell proliferation and inhibited apoptosis possibly via P65. (A) Confirmation of protein expression levels following si-P65 transfection by Western blotting in H1299 and PC-9 cells (B) CCK-8 assay was performed to detect tumor cell proliferation after si-P65 mix transfection in H1299 and PC-9 cells. (C) Flow cytometry was employed to detect the apoptosis percentages after si-P65 mix transfection in H1299 and PC-9 cells. (D) and (E) Confirmation of protein expression levels following si-P65 mix transfection by Western blotting in CSE1L overexpressed A549 and H292 cells. (F) and (G) Colony formation assay was performed in the CSE1L overexpressed A549 and H292 cells after si-P65 mix transfection. (H) and (I) Flow cytometry was employed to detect the apoptosis percentages in the CSE1L overexpressed A549 and H292 cells after si-P65 mix transfection. Error bars represent the SD. *P < 0.05; **P < 0.01.

Figure 6

CSE1L regulated P65 and promoted NSCLC cell proliferation and inhibited apoptosis by activating the NF- κ B/MAPK signaling pathway. (A) KEGG enrichment analysis was used to analyze the genes regulated by the down-regulation of CSE1L in H1299 cell. (B) and (C) Apoptosis subset and TNF signaling were enriched in GSEA. (D) and (E) Gene expression of selected genes from the apoptosis subset and TNF signaling detected by qRT-PCR assay in CSE1L knockdown H1299 cell. (F) Schematic diagram of the NF- κ B/MAPK signaling pathway. (G) and (H) Representative blots showing the protein levels of MAPK

signaling pathway in the CSE1L knockdown H1299 or overexpressed A549 cells. β -actin was used as an internal control.

Figure 7

CSE1L expression paralleled that of P65 and correlated with the poor prognosis of NSCLC patients. (A) Photos of representative blots for CSE1L and P65 expression in 40 pairs of lung cancer and noncancerous tissues. (B) The fold changes of CSE1L and P65 expression in lung cancer tissues

compared with noncancerous tissues. (C) The correlation between CSE1L and P65 expression in NSCLC tissues. (D) The protein levels of CSE1L and P65 and their correlation in NSCLC cells. (E) The correlation between CSE1L and P65 expression in 25 lung adenocarcinoma tissue microarrays. (F) The overall survival of P65 in GEO database GSE13213. (G) The overall survival of CSE1L and p65 in GEO database GSE13213.

Figure 8

Schematic model of the potential mechanism of CSE1L in NSCLC. CSE1L binds to the nuclear export signal (NES) on P65. The complex is subsequently docked to NPC and passes through the NPC into the cytoplasm. Hydrolysis GTP to GDP causes the disassembly of CSE1L-P65 complex and release of P65 and CSE1L in the cytoplasm, then activate the NF-κB/MAPK pathway, resulting in the promotion of cell proliferation and the inhibition of cell apoptosis.

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