High expression of CIN85 promotes proliferation and invasion of human esophageal squamous cell carcinoma

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Abstract. SH3 domain-containing kinase-binding protein 1 (CIN85), an 85 kDa protein known to be a member of the signal adaptor family, is abnormally expressed in several human malignancies and has been found to be involved in the growth, migration and invasion of these tumors. The objective of the present study was to clarify the clinical significance of CIN85 in human esophageal squamous cell carcinoma (ESCC), as well as its *in vitro* functions. CIN85 expression was evaluated in 129 cases of ESCC and its adjacent normal tissues using immunohistochemistry to explore its clinical relevance and prognostic value. The functions of CIN85 in the ESCC TE1 cell line were analyzed *in vitro* using the interfering short hairpin RNA silencing technique. MTS, wound healing, clone formation and Transwell assays were used to detect the proliferation, migration and invasion of ESCC cells. CIN85 expression was identified mainly in ESCCs and their adjacent normal tissues, and the high expression of CIN85 was significantly associated with advanced Tumor Node Metastasis stage and lymph node metastasis. CIN85 gene silencing significantly inhibited TE1 cell proliferation, migration and invasion. These results demonstrated that CIN85 was highly expressed in advanced stage ESCC and lymph node metastasis, and played a critical role in tumor proliferation and progression. Therefore, CIN85 may be a promising therapeutic target for human ESCC.

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Introduction

Esophageal cancer is the 8th most common human cancer and 6th highest in mortality (1). There are obvious regional and histological differences in the incidence of esophageal cancer. China has one of the highest incidence rates in the world, with the histological type of most patients being squamous cell carcinoma, which exceeds 90% of the total number of cases (2). Esophageal cancer is one of the most difficult gastrointestinal malignancies to treat and cure. Patients often experience distant metastasis or local disease recurrence, even after undergoing curative resection. Although multi-modality approaches based on surgery combined with preoperative chemotherapy and/or radiotherapy have been attempted, the efficacy of these treatments is limited, and overall survival remains poor (3-6). Therefore, novel strategies against esophageal cancer need to be developed and established to improve the prognosis of patients.

SH3 domain-containing kinase-binding protein 1 (CIN85) was first identified in human cells as a Cbl-interacting 85 kDa protein. CIN85 contains three Src homology 3 (SH3) domains at its N-terminus, followed by a proline-rich region and a C-terminal coiled-coil region (7). In association with casitas B-lineage lymphoma, an E3 ubiquitin ligase, CIN85 controls the intracellular internalization, trafficking and sorting of several activated receptor tyrosine kinases, including the epidermal growth factor receptor (EGFR) (8). Moreover, through the SH3 domains and the proline-rich region, CIN85 is implicated in a number of protein-protein interactions and has been found to play important roles in other processes, such as signal transduction, vesicle-mediated transport, cytoskeleton remodeling, immunological synapse, cell migration and invasion (9-11). Previously, CIN85 was detected on lamellipodia and invadopodia, which are involved in cell adhesion and migration, suggesting that the overexpression of CIN85 could promote the invasiveness of cancer cells (12,13).

However, the expression profiles and clinical relevance of CIN85 in esophageal squamous cell carcinoma (ESCC) remain unknown. The present study was designed to elucidate

the clinical significance of CIN85 in ESCC, as well as its *in vitro* functions.

Materials and methods

Patient cohort and cell line. A total of 129 patients were included in the present study, which was approved by the Institutional Ethics Committee of The First Affiliated Hospital of China Medical University (Shenyang, China). All patients were operated with curative intent between January 2014 and January 2017 at the Department of Thoracic Surgery, The First Affiliated Hospital of China Medical University. All patients were included except those who had undergone chemotherapy before the operation. The distance between cancer tissue and the adjacent normal tissue was >5 cm. A summary of the clinicopathological data is provided in Table I. No radiotherapy, chemotherapy or other adjuvant therapy was performed prior to surgery. Tumor staging in the present study was based on the 8th edition of the World Health Organization Tumor Node Metastasis (TNM) staging criteria for ESCC, published in 2016 (14).

A human ESCC TE1 cell line was obtained from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. Cells were routinely cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Cytiva) and 1% penicillin/streptomycin at 37˚C in 5% CO_2.

Immunohistochemistry (IHC) and scoring. IHC was performed on 4-μm thick paraffin sections fixed in 100% formaldehyde for 24 h at room temperature, which were then dewaxed and rehydrated in a descending ethanol series (100, 95, 85 and 75% for 5 min each at room temperature). For antigen retrieval, slides were placed in 0.01 ml/l citrate buffer in a pressure cooker on high pressure at 110-120˚C for 10 min. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 30 min at room temperature, and 5% goat serum (abs933; Shanghai Universal Biotech Co., Ltd.) was used for non-specific antibody blocking for 30 min at room temperature. Rabbit anti-human CIN85 monoclonal antibody (1:50; cat. no. ab151574; Abcam) was used overnight at 4˚C. Next, an anti-mouse/rabbit ready-to-use kit(cat. no. abs957; Shanghai Universal Biotech Co., Ltd.) and DAB kit (cat. no. 8059S; Shanghai Universal Biotech Co., Ltd.) were used according to the manufacturer's instructions. Finally, the slides were counterstained for 4 min with hematoxylin at room temperature.

All tissue slides were examined by two independent pathologists. The immunohistochemical results for the CIN85 protein were recorded by analyzing the average cells in five randomly selected high-power fields under a light microscope (magnification, x400). Positive cells were considered those whose cytoplasm or cell membrane was pale yellow to brown. The scoring was system was as follows: i) 0 points, no positive cells; ii) 1 point, <25% positive cells; iii) 2 points, 26-50% positive cells; iv) 3 points, 51-75% positive cells; and v) 4 points, >75% positive cells. Coloring intensity was sored as follows: i) 0 points, colorlessness; ii) 1 point, pale yellow; iii) 2 points, brown; and iv) 3 points, sepia. The sum of the two scores gave the final score, with scores 0-3 being negative expression and 4-7 being positive expression.

Immunocytochemistry assay. 200-μl cell suspension ($1x10^5$/ml) was added on coverslips in a 24-well culture plate and fixed with 4% paraformaldehyde for 15 min at room temperature. Fixed cells were washed three times with 1X PBS, then 5% goat serum (abs933; Univ,China) was used for non-specific antibody blocking for 30 min at room temperature and followed by overnight incubation with 2 μg/ml rabbit anti-human CIN85 monoclonal antibody (1:50; cat. no. ab151574; Abcam) at 4˚C. The cells were rinsed three times with PBS and incubated at room temperature with Alexa Fluor® 488-conjugated goat anti-rabbit IgG secondary polyclonal antibody (1:1,500; cat. no. 150077; Abcam) for 1 h. The cells were rinsed three times with PBS, then counterstained with Fluoroshield™ containing DAPI Staining Solution (cat. no. C1005; Beyotime Institute of Biotechnology). The slides were examined under an immunofluorescence microscope (Olympus FV-100) at x200 magnification.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted using the miRNeasy Mini kit (Qiagen AB), according to the manufacturer's instructions. cDNA was synthesized using 1 μg extracted mRNA as the template with a GoScript™ Reverse Transcription kit (Promega Corporation), according to the manufacturer's instructions. Primer sequences for CIN85 and GAPDH were as follows: CIN85 sense, 5'-ACG ATCAGCGTGGGTGAAAT-3' and antisense, 5'-CGCTCG CCTCTCTTATTGGT-3'; GAPDH sense, 5'-AAGAGCACA AGAGGAAGAGAGAGAC-3' and antisense, 5'-GTCTACAT GGCAACTGTGAGGAG-3'. mRNA expression levels were quantified using the RT^2 SYBR Green qPCR Master Mix (Promega Corporation) and detected using the 7900HT Fast Real-Time PCR system (Thermo Fisher Scientific, Inc.). The qPCR mix contained 0.4 μl of each primer, 10 μl RT^2 SYBR Green qPCR Master Mix and 2 μl cDNA. Nuclease-free water was added to achieve a final reaction volume of 20 μl. The qPCR reaction condition was set to 95˚C for 2 min, followed by 40 cycles of 95˚C elongation for 15 sec and 60˚C for 1 min each. A melting curve was then calculated for each PCR product to confirm synthesis specificity (15).

Protein extraction and western blotting. Cells were harvested and lysed using lysis buffer (Cell Signaling Technology, Inc.) with 1 mM PMSF (Beyotime Institute of Biotechnology). The concentration of total protein was measured using the Pierce™ BCA Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Protein aliquots (20 μg) were loaded with SDS buffer (Beyotime Institute of Biotechnology) and boiled at 95˚C for 10 min. The denatured protein samples were then subjected to western blotting. Same amounts of protein samples were isolated by 12% SDS-PAGE gels, then transferred onto PVDF membranes. The membranes were then blocked with TBS-T containing 5% non-fat milk powder for 2 h at room temperature and incubated with primary antibodies at 4˚C overnight. The membranes were then incubated with secondary antibody for 1 h at room temperature. Rabbit anti-human CIN85 monoclonal (1:200; cat. no. ab151574; Abcam) and anti-human GAPDH monoclonal (1:500; cat. no. ab181602; Abcam) primary antibodies were used. Then, membranes were incubated with a HRP-conjugated

Table I. Association between the expression of CIN85 and the clinicopathological features of patients with esophageal squamous cell carcinoma.

Clinical characteristic	Number of cases	CIN85 expression		χ^2	P-value
		Negative, n (%)	Positive, n (%)		
Sex				0.548	0.459
Male	102	30 (29.4)	72 (70.6)		
Female	27	6 (22.2)	21 (77.8)		
Age, years				0.568	0.451
<60	72	22 (30.6)	50 (69.4)		
≥60	57	14 (24.6)	43 (75.4)		
Tumor invasion, T				0.520	0.471
T1-T2	87	26 (29.9)	61 (70.1)		
T3-T4	42	10 (23.8)	32 (76.2)		
Lymph node metastasis, N				11.356	0.001
N0	66	27 (40.9)	39 (59.1)		
N1-N3	63	9 (14.3)	54 (85.7)		
Cell grading				0.000	0.989
G1-G2	111	31 (27.9)	80 (72.1)		
G3	18	5 (27.8)	13 (72.2)		
TNM stage				9.768	0.002
I-II	72	28 (38.9)	44 (61.1)		
III-IV	57	8 (14.0)	49 (86.0)		

CIN85, SH3 domain-containing kinase-binding protein 1; TNM, Tumor Node Metastasis.

goat anti-rabbit IgG polyclonal secondary antibody (1:500; cat. no. 7074; Cell Signaling Technology, Inc.). Antibodies were all diluted according to the manufacturer's instructions. Western blotting was performed as previously described (16). The grayscale values of the resulting bands were measured using ImageJ (version 1.46r) software (National Institutes of Health).

Lentivirus construction and cell transduction. Lentiviral vectors were cloned and packaged with the GV248 plasmid, which contained a target interfering short hairpin RNA (shRNA), Hepler1.0 and Helper2.0. The shRNA targeting human CIN85 (5'-AAGACCAGAAATGCTTCCAAA-3') and the negative control shRNA (5'-TTCTCCGAACGTGTCA CGT-3') were designed, synthesized and inserted into the GV248 plasmid by Shanghai GeneChem Co. Ltd. The second-generation system was used. 293T cells were purchased from the American Type Culture Collection and were used as the interim cell line. The mass of lentiviral plasmid used was 20 μg and the ratio of the lentiviral plasmid, packaging vector and envelope vector was 4:3:2. After 15 days of the lentivirus construction, when the cells grew to 70-80% confluence, TE1 cells were infected with different titers ($5x10^8$ U/ml, $6x10^8$ U/ml and $1x10^9$ U/ml) of the virus in the presence of 5 μg/ml Enhanced Infection Solution and polybrene (Shanghai GeneChem Co., Ltd.). The medium was changed 12 h later. After 48 h of cell transduction, the most suitable multiplicity of infection (70%) was determined by observing the minimum lentivirus and the relatively largest number of fluorescent cells. Puromycin (Merck KGaA) was used at 1 μg/ml to screen for TE1 cells that were successfully transduced.

MTS assay. An MTS assay (Promega Corporation) was performed to assess the cell viability of TE1 cells, according to the manufacturer's instructions. In brief, logarithmic growth phase cells were suspended and diluted to $1x10^4$/ml with medium and inoculated at 200 μl per well in 96-well plates. Cell viability was then measured by detecting the absorbance at 490 nm on days 0, 1, 2, 3, 4 and 5. Each experiment was conducted in triplicate.

Clone formation assay. A total of 200 cells per well were inoculated into 6-well plates and allowed to grow under conditions of 37˚C with 5% CO_2 for 2 weeks. The medium was changed every other day. The clones (≥50 cells) were counted under a light microscope (magnification, x400) after fixation with 4% paraformaldehyde for 15 min and staining with crystal violet solution for 5 min at room temperature.

Wound healing assay. When cells grew to 80-90% confluence (~$1x10^6$ cells per well) in 6-well plates, sterilized 1-ml tips were used to generate wounding across the cell monolayer, and the debris was washed with PBS. The medium was then replaced with serum-free medium. Cells were observed and photographed in three fields randomly selected from each well under

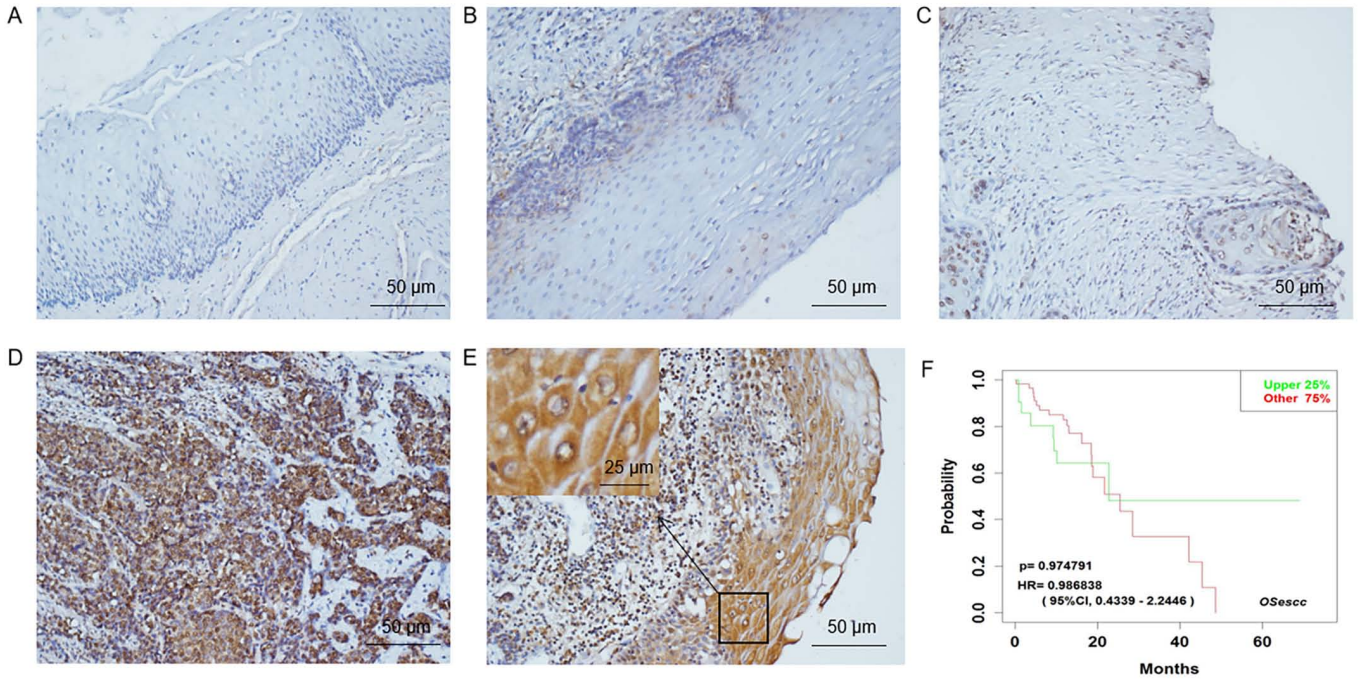

Figure 1. Immunohistochemical staining of CIN85 in esophageal squamous cell carcinoma and adjacent normal tissues. (A) Negative expression of CIN85 in adjacent normal tissue cells. (B) Low expression of CIN85 in the basal cells of the epithelium in adjacent normal tissue cells. (C) Negative expression of CIN85 in cancer tissue cells. (D and E) Positive expression of CIN85 in cancer tissue cells. Magnification, x200. (F) Kaplan-Meier survival curve showing the relationship between CIN85 expression and overall survival. CIN85, SH3 domain-containing kinase-binding protein 1.

an inverted microscope (magnification, x200) at 0, 12 and 24 h. Wound areas were measured at 0 and 24 h using ImageJ (version1.46r; National Institutes of Health). The migration rate was then calculated as (wound area at 24 h migration/wound area at 0 h. Each experiment was conducted in triplicate.

Transwell invasion assay. Cell suspension was prepared at a density of $1x10^5$/ml in a serum-free medium. A 200-μl cell suspension was added to the upper Transwell chamber (Corning, Inc.) with an insert coated with Matrigel™ (1:8; BD Biosciences) for 30 min at 37˚C for the invasion assay. Complete medium (500 μl) containing 10% fetal bovine serum was added to the lower chamber. After 24 h of cultivation at 37˚C, the Matrigel and cells on the upper chamber were gently wiped off with a cotton swab. The Transwell membranes were cut off using a surgical blade and stained with crystal violate dye for 5 min at room temperature. For quantification, the integral optical density (IOD) of the two groups was measured under a light microscope under low magnification (magnification, x4).

Statistical analysis. Statistical analysis was performed with SPSS software 24.0 (IBM Corp.) and GraphPad Prism 6.0 software (GraphPad Software, Inc.). The differential expression of CIN85 and its association with clinicopathological factors was analyzed by χ^2 and Fisher's exact tests. Quantitative data are expressed as the mean ± SD and were analyzed by Student's t-test. The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/) was screened using the 'esophageal carcinoma' and 'CIN85/SH3KBP1' search terms. The patients with upper 25% CIN85 expression were considered as the high-expression group. Kaplan-Meier survival curves were constructed using The Biomedical Informatics Institute OSescc tool (bioinfo.henu.edu.cn/DBList.jsp) (17) and hazard ratio (HR) and log-rank P-value were calculated. $P<0.05$ was considered to indicate a statistically significant difference.

Results

CIN85 is differentially expressed in ESCC and non-neoplastic esophageal tissue. A total of 129 patients with ESCC were included in the present study. In 54 of the adjacent normal tissue specimens, CIN85 was found to be positively expressed in the basal cells of the esophageal epithelium in the adjacent normal tissue specimens. The remaining 75 normal samples showed negative expression of CIN85. Among the cancer specimens, 93 were positive and 36 negative (Fig. 1A-E), corresponding to a positive rate of 72.1%. CIN85 was primarily expressed in the cell membrane and cytoplasm (Fig. 2).

Increase in CIN85 expression is associated with advanced tumor stage and metastatic disease. To evaluate the potential role of CIN85 in ESCC, TCGA was searched, and a total of 29 patients were included. The OSescc tool for ESCC prognosis analysis was used to plot a Kaplan-Meier survival curve (Fig. 1F). There was no significant difference in the overall survival rate between patients with low and high CIN85 expression. Table I summarizes the association between the expression of CIN85 and the clinicopathological features of patients with ESCC. It was found that CIN85 was highly expressed in patients with advanced TNM stage (P=0.002) and those with lymph node metastasis (P=0.001) (Table I).

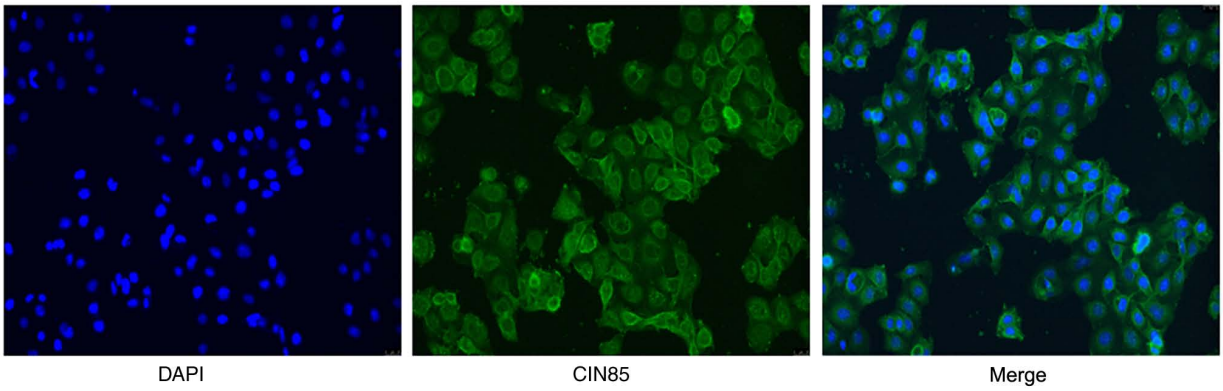

Figure 2 Immunocytochemistry staining assay. A primary antibody against CIN85 was used in the TE1 cell line (green), and then merged with DAPI staining (blue) at x200 magnification. CIN85 is mainly expressed in the cell membrane and cytoplasm. CIN85, SH3 domain-containing kinase-binding protein 1.

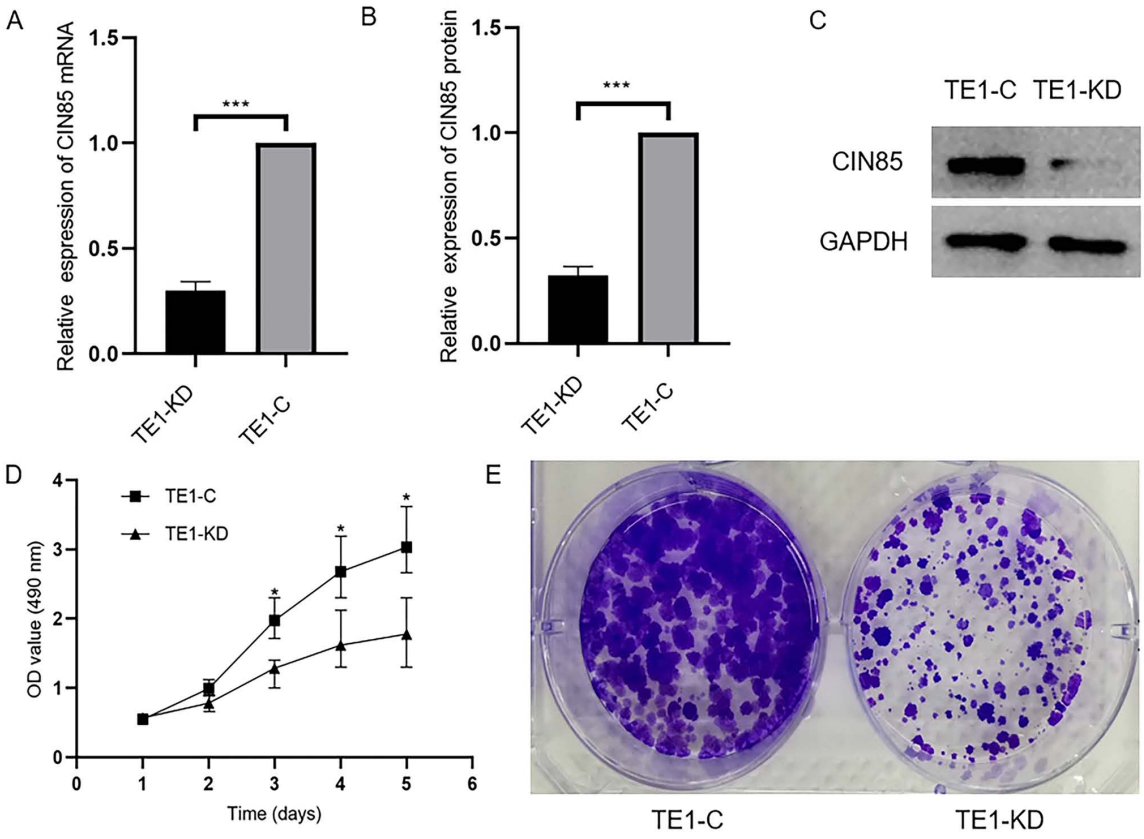

Figure 3. Verification of specific shRNA transfection. (A) Relative expression of CIN85 mRNA. The mRNA expression levels of CIN85 were decreased significantly in TE1-KD cells, as compared with the TE1-C group. (B) Relative expression of CIN85 protein. The protein expression levels of CIN85 were decreased significantly in TE1-KD cells, as compared with the TE1-C group. (C) CIN85 expression in the two groups as detected by western blotting. (D) MTS and (E) clone formation assays showed that the proliferation of esophageal squamous cell carcinoma cells was significantly decreased in the TE1-KD cells. *P<0.05 and ***P<0.001 vs. TE1-KD. shRNA, small hairpin RNA; CIN85, SH3 domain-containing kinase-binding protein 1; KD, knockdown; C, negative control.

Transfection and verification of CIN85-specific interfering RNA. In order to study the effect of CIN85 on the biological behavior of ESCC cells, the TE1 cell line was infected with a virus containing a specific shRNA and a negative control virus, respectively designated as TE1-KD and TE1-C cells. Fig. S1 demonstrates that a high frequency of green fluorescent ESCC cells was observed 48 h after transfection, suggesting successful transfection. Next, RT-qPCR and western blotting were performed to detect transfection efficiency. The mRNA and protein expression levels of CIN85 were decreased significantly in TE1-KD cells, as compared with the controls (Fig. 3A-C).

Downregulation of CIN85 can inhibit cell proliferation. After the successful construction of the TE1-KD cell and TE1-C cell group, MTS and monoclonal formation assays were

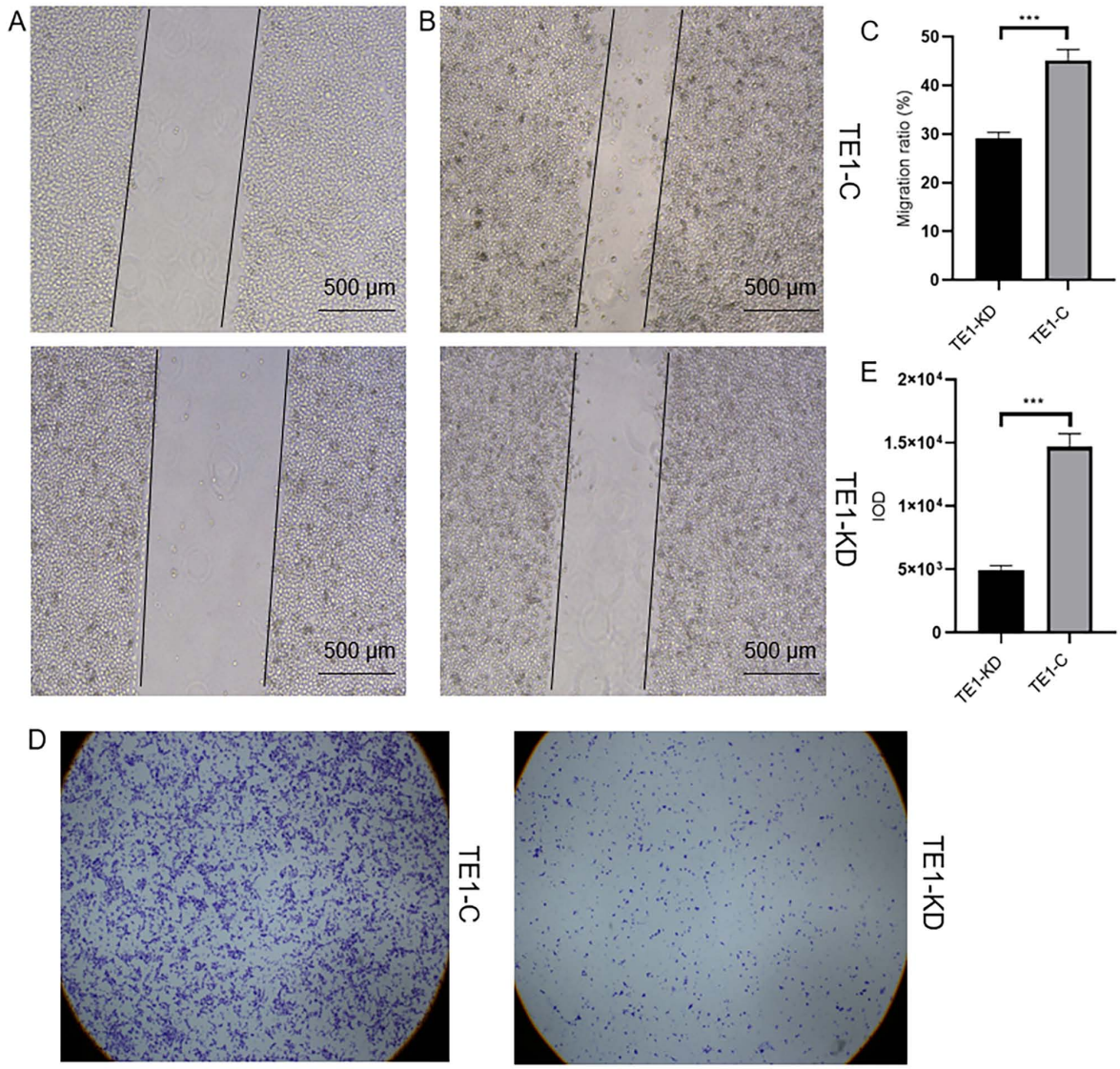

Figure 4. Effect of CIN85 on the migration and invasion of TE1 cells. Wound healing assay of TE1-C and TE1-KD cells at 0 h (A) and 24 h (B) (scale bar, 500 μm). (D) Transwell assay. (C and E) Migration ratio and IOD in the TE1-KD group was significantly decreased, as compared with that in the control group. ***P<0.001. CIN85, SH3 domain-containing kinase-binding protein 1; KD, knockdown; C, negative control; IOD, integral optical density.

conducted. It was found that the proliferation of ESCC cells was significantly decreased following the downregulation of CIN85 (Fig. 3D and E).

Downregulation of CIN85 can inhibit cell invasion. In an attempt to explore the effect of CIN85 on TE1 cell invasion, the migration ratio and IOD were assessed by wound healing and Transwell assays, respectively. Migration ratio and IOD in the TE1-KD group was significantly decreased, as compared with that in the control group (TE1-KD 29.06±1.315 vs. TE1-C 45.14±2.275, P<0.001; TE1-KD 4,866±320.8 vs. TE1-C 14,671±2,039, P<0.001; Fig. 4), suggesting that the downregulation of CIN85 can inhibit cell migration and invasion.

Discussion

Esophageal cancer is one of the most common malignant tumors of the digestive tract. ESCC is the main pathological type of esophageal cancer in China (2). At present, the primary treatment of esophageal cancer is a comprehensive treatment that is based on surgical treatment, combined with chemotherapy, radiotherapy, chemoradiotherapy or immunotherapy (3). Although the cure rate of early esophageal cancer through endoscopic surgery has reached >90%, early esophageal cancer was diagnosed less frequently (4). Additionally, the poor 5-year survival rate and high recurrence of advanced esophageal cancer remains a problem (18). With the introduction and development of individualized treatment, further studies are required to identify effective therapeutic targets for esophageal cancer. EGFR has been found to be highly expressed in 42.5-85.7% of ESCC cases and is closely associated with the recurrence and poor prognosis of esophageal cancer (19). Tyrosine kinase receptor inhibitors have been used in combination with chemotherapeutic drugs, and inhibitors of vascular EGFR in combination with chemotherapy for the treatment of esophageal cancer; however, the two aforementioned agents have not achieved outstanding results (20). The mechanism of resistance among them is not yet clear, so

knowledge of the precise expression pattern and characterization of esophageal cancer is required to further explore novel therapeutic targets. The CIN85 gene is located on the distal end of the human X chromosome and was first discovered in human gliomas (21). Previous studies have found that there are >100 proteins that interact with the CIN85 adaptor protein, of which the tyrosine receptor kinase is a major class (22-24). CIN85 accomplishes downstream signal transduction by mediating tyrosine receptor kinase endocytosis and the trafficking of its vesicles. Abnormal endocytosis and transport cause pathological changes in cells (25). Other studies have demonstrated that CIN85 is involved in the formation of cell membranes and the cytoskeleton, and is associated with the remodeling of the cytoskeleton, thereby promoting the invasion and metastasis of tumor cells (26,27). Nam *et al* (28) demonstrated that the CIN85 complex is a component of the invasive machinery of pseudotumor breast cancer cells, and is directly linked to malignant behavior. Another study revealed that the CIN85 adaptor protein could be directly associated with the proto-oncogene H-ras (29).

A number of studies have found that CIN85 is highly expressed in colon and breast cancer, oral squamous cell carcinoma, glioma, melanoma, and other cancer tissues (11-13,30), but at present no study has investigated the role of CIN85 in ESCC. The present study found that CIN85 was highly expressed in patients with advanced TNM stage and those with lymph node metastasis, suggesting a poor prognosis, which was consistent with other previous studies. On this basis, the present study further constructed CIN85-knockdown cell lines and found that the proliferation, migration and invasion of ESCC cells were significantly inhibited in the CIN85 knockdown group. This indicated that CIN85 could promote the proliferation and metastasis of ESCC cells, and was directly associated with malignant behaviors, such as tumor recurrence, and as a consequence, affects the prognosis of patients.

However, the major limitation of this research was that the effects of CIN85 on the proliferative and migratory activities of ESCC cells were only confirmed in one ESCC cell line. Cell experiments were actually conducted with three cell lines, Kyse 30, Kyse 350 and TE 1, and two knockdown sequences were designed for each cell type. However, the knockdown of CIN85 expression failed in the Kyse 30 (Fig. S2) and Kyse 350 (data not shown) cell lines, so only one cell line was used in the subsequent studies. It was speculated that the different cell types caused the failure. Although the three cell lines are all ESCC cell lines, TE1 is a low differentiated ESCC cell line, whereas Kyse 350 is medium differentiated and Kyse 30 is high differentiated. To the best of our knowledge, this is the first report to investigate the abnormal expression of CIN85 in ESCC, so further research in this field are encouraged to demonstrate that the present results are not accidental.

CIN85 plays a role in multiple tumors, mainly through its N-terminal SH3 domain interacting with other proteins, so the SH3 domain may be the most promising research target (31). At present, there have been numerous signs of progress in preventing the development of tumor cells by focusing on the SH3 domain. In particular, a previous study by Hashimoto *et al* (32), used peptide ligands targeting SH3 not only *in vitro* but also *in vivo* to successfully reduce the invasion and metastasis of breast cancer without significant adverse events. Sato *et al* (33) also demonstrated that the inhibition of the SH3 domain of CIN85 using a lysyl oxidase precursor peptide could reduce the degradation of the surrounding matrix and decrease the invasive and metastatic ability of breast cancer cells. These studies indicated that the use of certain molecules to block the SH3 domain of CIN85 can, in principle, serve as a basis for the study of novel antitumor drugs.

In conclusion, the present study provided possible target genes for basic and clinical studies of ESCC. CIN85 is closely associated with the growth and migration of ESCC and may be an effective target for the treatment of esophageal cancer. However, the occurrence and development of tumors is a multi-factor and multi-stage process. Therefore, the specific underlying mechanism of CIN85 involved in the occurrence and development of esophageal cancer requires further study.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SGZ and XYH designed the research. XYH, XXB and XC performed the research and analyzed the results. SGZ and XYH wrote the paper. XXB and XC edited the manuscript and provided critical comments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Ethics Committee of The First Affiliated Hospital of China Medical University (Shenyang, China). Patients provided written informed consent.

Patient consent for publication

All patients provided written informed consent for the publication of any associated data and accompanying images.

Competing interests

The authors declare that they have no competing interests.

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