Upregulation of KIF18B facilitates malignant phenotype of esophageal squamous cell carcinoma by activating CDCA8/mTORC1 pathway

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Abstract

Background: Kinesin family member 18B (KIF18B) has been regarded as an oncogene that is abnormally overexpressed in some cancers, but its mechanism in esophageal squamous cell carcinoma (ESCC) remains unclear, which is thereby investigated in this study.

Methods: Bioinformatics analysis was performed to analyze the expression of KIF18B in esophageal carcinoma (ESCA). Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect KIF18B expression in ESCC cells. After KIF18B overexpression or cell division cycle associated 8 (CDCA8) deficiency, ESCC cells were subjected to determination of qRT-PCR, Western blot, cell counting kit-8 assay, flow cytometry, wound healing, and Transwell assay. The mechanism of KIF18B in the mechanistic target of rapamycin complex 1 (mTORC1) pathway was detected by Western blot.

Results: KIF18B was overexpressed in ESCA samples and ESCC cells. Upregulation of KIF18B enhanced the viability, accelerated cell cycle by elevating CDK4 and Cyclin D3 levels as well as promoted the migration and invasion by decreasing E-cadherin level and increasing Vimentin and N-cadherin levels in ESCC cells, which was counteracted by CDCA8 silencing. The expression of CDCA8 in ESCC cells was upregulated by KIF18B overexpression. KIF18B overexpression activated the mTORC1 pathway by upregulating phosphorylated (p)-/p70S6K and p-/mTOR levels in the ESCC cells, which was reversed by CDCA8 silencing.

Conclusion: KIF18B overexpression promotes the proliferation, migration, and invasion of ESCC cells via CDCA8-mediated mTORC1 signaling pathway in vitro.

Keywords
cell division cycle associated 8, esophageal squamous cell carcinoma cells, kinesin family member 18B, malignant phenotype, mechanistic target of rapamycin complex 1 pathway

Jiangliu Xie and Bo Wang contributed equally to this work.

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1 | INTRODUCTION

Esophageal cancer is the seventh most common malignant tumor in the world and the sixth most leading cause of cancer-related death.\(^1\) China is a country with a high incidence of esophageal cancer, with esophageal squamous cell carcinoma (ESCC) as the main pathological type. More than half of the ESCC patients worldwide are in China. Currently, ESCC is the fourth most common and fourth leading cause of cancer-related death.\(^2\) ESCC belongs to gastrointestinal tumor and is characterized by insidious onset, rapid progression, metastatic tendency, and high malignancy, 70% of patients with which are diagnosed at an advanced stage in China.\(^3,4\) Existing first-line treatment for ESCC has a poor prognosis, and there are few second-line treatment options for patients with advanced or metastatic ESCC who are progressing or intolerant to first-line standard chemotherapy.\(^5–8\) Therefore, seeking a biomarker with both high sensitivity and high specificity is of significance for the diagnosis and prognosis of ESCC.

Kinesin families (KIFs) constitute a class of protein superfamilies that belong to motor proteins, which is responsible for transporting different molecular cargoes along microtubules to specific intracellular locations in the cells.\(^9\) Kinesins have been found to be widely distributed in various tissues and cells, playing an important role in maintaining the normal morphology and function of cells.\(^10\) Much has been learned about the biological functions of kinesins, and studies have demonstrated that KIFs are closely related to the development of neurodegeneration, diabetes, kidney disease, etc.\(^11–13\) In recent years, mounting research studies have been carried out on KIFs in tumors.\(^14\) A large body of evidence has suggested that KIFs are widely involved in the development of many tumors, such as hepatocellular carcinoma, gastric cancer, and cervical cancer, and that changes in their expression levels are directly related to tumorigenesis.\(^15–17\)

Kinesin family member 18B (KIF18B) belongs to the kinesin 8 family among 14 kinesin subfamilies, which is closely associated with the depolymerization of astral microtubules during cell mitosis.\(^18\) At present, KIF18B has been shown to be highly expressed in several cancers.\(^19–21\) A recent study indicates that KIF18B promotes the proliferation of pancreatic ductal adenocarcinoma by activating the expression of cell division cycle associated 8 (CDC8A).\(^22\) Additionally, it has been found that CDC8A regulation is associated with the mechanistic target of rapamycin complex 1 (mTORC1) signaling in ESCC.\(^23\) Moreover, downregulation of the mTORC1 signaling has been found to inhibit the progression of ESCC in some reports.\(^24,25\) Currently, although a few other kinesins have been studied in ESCC, the mechanism of KIF18B in ESCC is poorly understood.\(^26,27\)

Therefore, this study intends to explore the mechanism of KIF18B in growth, migration, and invasion of ESCC and figure out whether the effect of KIF18B is realized by the CDC8A-mediated mTORC1 signaling pathway.

2 | MATERIALS AND METHODS

2.1 | Bioinformatics analysis

The expression level of KIF18B in esophageal carcinoma (ESCA) (tumor = 182, normal = 286) was predicted by GEPIA (http://gepia2.cancer-pku.cn/).

2.2 | Cell treatment and transfection

Human normal esophageal epithelial cell line (HEEC, BNCC337729) and ESCC cell lines EC9706 (BNCC352127) as well as KYSE30 (BNCC339894) were obtained from BeiNa Culture Collection (Beijing, China). Another two cancer cell lines KYSE220 (JCRB1086) and KYSE150 (JCRB1095) were provided by Japanese Collection of Research Bioresources (JCRB). All cells were cultured using Ham’s F12 medium (PM150810, Procell, China) containing 2% fetal calf serum (164210, Procell, China) in the environment of 37°C with 5% CO₂.

As for cell transfection, the plasmid equipped with full-length human KIF18B was synthesized by Genechem, and pcDNA3.1 vectors (VT1001, YouBio, China) were used to construct overexpression plasmids and corresponding controls. Then, the transfection was conducted with Lipofectamine RNAiMAX Transfection Reagent (13778-075, Invitrogen, USA), in keeping with the protocol. After 72h of incubation, transfection efficiency was estimated using quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot.

2.3 | Quantitative real-time polymerase chain reaction

Total RNA from ESCC cells was extracted using TRIzol reagent (15596-026, Invitrogen, USA). The cDNA Synthesis Kit (D7168M, Beyotime, China) was then performed for reverse transcription. Afterwards, the cDNA template was amplified using PCR Master Mix (D7255, Beyotime, China) on a real-time PCR system (7500, Applied Biosystems, USA). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a loading control. Indicated gene expressions were analyzed using the 2−ΔΔCt method.\(^28\) All sequences of primers were listed below:

**KIF18B**, forward: 5’-GCTGAACTTAGTGATACGGG-3’; reverse: 5’-CCTGAGGGTTAAACACCAGCA-3’.

**CDC8A**, forward: 5’-TTGACTACTTCGCCCTTG-3’; reverse: 5’-CTTCTTCTTCCTCTCCACTA-3’.

**GAPDH**, forward: 5’-ACCAGAYCCATGCCATCAC-3’; reverse: 5’-TCCACCACCCTGTTGCTGTA-3’.

2.4 | Western blot

Total proteins from ESCC cells were extracted using RIPA Lysis Buffer (P0013C, Beyotime, China) containing Phosphatase Inhibitor
(abs9162, Absin, China), The BCA Protein Assay Kit (P0011, Beyotime, China) was used for protein quantification. Afterward, the lysate was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto the polyvinylidene fluoride membrane (IPVH00010, Millipore, USA) and ultimately blocked using BLOT-QuickBlocker (C006011-0175, Sangon Biotech, China) at room temperature for 60 min with gentle shaking. Subsequently, the separated proteins were reacted with primary antibodies at 4°C overnight, followed by being incubated with the secondary antibody Goat Anti-Rabbit IgG (ab6721, Abcam, UK, 1:2000) or Rabbit Anti-Mouse IgG (ab6728, Abcam, UK, 1:2000) at room temperature for 1 h. At last, the enhanced chemiluminescence system (17-373BP, Millipore, USA) was introduced to visualize immunoreactive proteins, and Image J software (vision 1.8.0, National Institutes of Health, USA) was applied to analyze protein bands. The primary antibodies adopted here encompassed those against KIF18B (ab168812, 94 kDa, Abcam, UK, 1:2000), CDK4 (ab108357, 34 kDa, Abcam, UK, 1:1000), CyclinD3 (ab28283, 35 kDa, Abcam, UK, 1:1000), p70S6K (ab32529, 70 kDa, Abcam, UK, 1:5000), phosphorylated (p)-p70S6K (ab59208, 60 kDa, Abcam, UK, 1:1000), p-mTOR (ab109268, 289 kDa, Abcam, UK, 1:1000), mTOR (ab134903, 289 kDa, Abcam, UK, 1:10,000), E-Cadherin (ab40772, 97 kDa, Abcam, UK, 1:10000), Vimentin (ab92547, 54 kDa, Abcam, UK, 1:1000), N-Cadherin (ab18203, 130 kDa, Abcam, UK, 1:1000), CDC42 (ab74473, 38 kDa, Abcam, UK, 1:1000), and GAPDH (ab8245, 36 kDa, Abcam, UK, 1:2000).

2.8 Statistical analysis

Data were analyzed by GraphPad Prism (vision 8.0, GraphPad, USA) and were presented as the mean ± standard deviation. All experiments were carried out independently for three times. The comparison among multiple groups was tested by the one-way analysis of variance (ANOVA). Value with p < .05 was perceived as statistically significance.

3 RESULTS

3.1 KIF18B was highly expressed in ESCC cells

GEPIA database predicted that significant upregulation of KIF18B existed in ESCA (Figure 1A, p < .05). In order to figure out the expression of KIF18B in ESCC, we performed qRT-PCR to detect its expression level in vitro. The results demonstrated that the expression of KIF18B in ESCC cell lines (EC9706, KYSE-220, KYSE150 and KYSE30) also presented at a high level when compared with that in HEEC cells (Figure 1B, p < .001).

3.2 Overexpression of KIF18B promoted ESCC cell viability and cell cycle

Given the results that KIF18B was relatively lower expressed in EC9706 and KYSE220 cells, we transfected KIF18B overexpression plasmid into these two cells for exploring the effect of KIF18B on tumorigenic phenotypes. Thereafter, the transfection efficiency of KIF18B overexpression plasmid was examined by both qRT-PCR and Western blot. As shown in Figure 1C-E, KIF18B expression was remarkably increased in KIF18B overexpression plasmid-transfected EC9706 and KYSE220 cells (p < .01). Next, CCK-8 assay was carried out to determine the cell viability, we found that KIF18B

2.6 Cell cycle detection

The cell cycle after transfection of KIF18B overexpression plasmid was assessed by a flow cytometer (CytoFLEX, Beckman, USA). In short, the transfected cells were cultured until its confluence is over 70%. Subsequently, cells were collected, fixed with 70% ethyl alcohol (459836, Sigma-Aldrich, USA) for 24 h, and then stained with propidium iodide (ST511, Beyotime, China) at 37°C for 30 min. Then, the flow cytometer was employed for calculation and analysis.

2.7 Cell migration and invasion detection

In order to detect the impact of KIF18B or CDCA8 upon migratory and invasive abilities of ESCC cells, wound healing assay and Transwell assay were performed. For wound healing assay, the transfected cells were inoculated in a 6-well plate until the cell confluence reached about 80%. Next, a long scrape was created with a sterile pipette tip, and phosphate buffered saline (PBS) was used to remove detached cells on the surface. Then, the cells were incubated in the Ham’s F12 medium for 48 h. Afterward, the wounds were photographed at 0 and 48 h by a microscope (x100 magnification). As for cell invasion detection, 3 x 10³ cells were suspended in the upper Transwell chamber (3428, Corning, USA) with precoated Matrigel (BD Bioscience) and filled with 200 µl Ham’s F12 medium. The lower chamber was added with 500 µl Ham’s F12 medium containing 10% FBS. Twenty-four hours of incubation later, we removed the cells in the upper chamber and then used 4% paraformaldehyde (E672002-0100, Sangon Biotech, China) to fix the invaded cells. Subsequently, these invaded cells were further stained by crystal violet (G1061, Solarbio, China) for 15 min, followed by being observed and counted using the microscope (x200 magnification).
overexpression enhanced the viability of EC9706 and KYSE220 cells (Figure 1F, G, p < .05). We also compared the cell cycle changes between KIF18B overexpression and its vector groups in both cells. Interestingly, the evaluation of cell cycle by flow cytometer assays indicated that the percentage of cell cycle in the G0/G1 phase was dropped in both cells after transfection, suggesting the acceleration of cell cycle caused by upregulation of KIF18B (Figure 2A–C, p < .05). For consolidating our findings, we measured cell-cycle-related protein levels by Western blot and found that CDK4 and CyclinD3 levels in both cells were evidently upregulated by KIF18B overexpression (Figure 2D–G, p < .01).

3.3 Overexpression of KIF18B enhanced the migratory and invasive abilities of ESCC cells

We subsequently investigated whether KIF18B overexpression affects the migratory and invasive abilities of ESCC cells. From the results illustrated in Figure 3A–C, we noticed that cell migration was greatly enhanced following the transfection of KIF18B overexpression plasmid (p < .01). Likewise, Transwell assays for detecting cell invasion revealed the similar outcomes, KIF18B overexpression promoted the invasion of ESCC cells (Figure 3D–F, p < .001). To confirm our previous findings, detailed evidence was necessary. Given that cell migration and invasion were closely associated with epithelial mesenchymal transition (EMT), we subsequently evaluated EMT-related protein levels. As a result, overexpression of KIF18B in ESCC cells signally downregulated E-cadherin level, yet conspicuously upregulated Vimentin and N-cadherin levels (Figure 4A–D, p < .01).

3.4 Overexpression of KIF18B upregulated CDCA8 and activated the mTORC1 pathway

On the ground of above findings, we focused on digging out the relationship between KIF18B and CDCA8 in ESCC as well as the mTORC1 pathway. As results of Western blot shown in Figure 4E–K, in cells transfected with KIF18B overexpression plasmid, the protein
level of CDCA8 was remarkably upregulated, and the mTORC1 pathway was evidently activated through highly elevating phosphorylation levels of p70S6K and mTOR (p<.01).

3.5 CDCA8 silencing reversed the effect of KIF18B overexpression on ESCC progression and inhibited the mTORC1 pathway

A few reports showed that CDCA8 was related to the mTORC1 signaling in some cancers, whereas there was little elucidation regarding the molecular mechanism of CDCA8 in ESCC cells transfected with KIF18B overexpression plasmid. Therefore, cells were transfected with siCDCA8 for follow-up rescue experiments. The downregulation of CDCA8 in EC9706 and KYSE220 cells was determined by Western blot and qRT-PCR, respectively. Compared with the cells transfected with KIF18B overexpressed plasmid and vector, the protein and miRNA expressions of CDCA8 were significantly reduced in both cells with co-transfection of KIF18B overexpressed plasmid and siCDCA8 (Figure 5A–F, p<.01). As shown in Figure 5G–J, the detection of cell-cycle-related protein levels revealed that siCDCA8 partially reversed the facilitating effects of KIF18B on CDK4 and Cyclin D3 levels in both cells (Figure 5G–J, p<.05). Expectedly, results of wound healing assay and Transwell assays also indicated that the migratory...
FIGURE 3 The effect of KIF18B overexpression on ESCC cell migration and invasion. Both EC9706 and KYSE220 cells were transfected with KIF18B overexpression plasmid. (A–C) The comparison on migratory ability of ESCC cells was evaluated by wound healing assay. Original magnification, 100x. Scale bar: 50 μm. (D–F) Transwell assays were performed to detect the invasion of the transfected cells. Original magnification, 200x. Scale bar: 50 μm. All experiments had gone through three repetitions.

* p < .05, ** p < .01, *** p < .001 versus Vector. ESCC, esophageal squamous cell carcinoma; KIF18B, kinesin family member 18B.
The effects of KIF18B overexpression on EMT-related proteins and mTORC1 pathway-related proteins in ESCC cells. Both EC9706 and KYSE220 cells were transfected with KIF18B overexpression plasmid. (A-D) Western blot was employed to examine the expression of EMT biomarkers, including E-cadherin, N-cadherin and Vimentin. GAPDH served as a loading control. (E-K) The levels of CDCA8 and mTORC1 pathway-related proteins (p-p70S6K, p70S6K, p-mTOR, and mTOR) were detected by western blot. GAPDH served as a loading control. All experiments had gone through three repetitions. **p < .01, ***p < .001 versus Vector. 

**DISCUSSION**

Based on the current circumstances that increasingly kinesins have been found to play a vital role in the progression of various cancers, we determined to explore the biological function and mechanism of KIF18B in ESCC. In the current study, we confirmed that the upregulation of KIF18B promoted proliferation and invasive abilities (Figure 6D–F, p < .05) of KIF18B overexpression plasmid-transfected cells were partially restrained by CDCA8 silencing. Furthermore, we verified the role of CDCA8 in the process of KIF18B regulating the mTORC1 pathway. The rescue experiments showed that siCDCA8 reversed the promoting effect of KIF18B on the mTORC1 pathway by downregulating levels of p-p70S6K and p/mTOR (Figure 7A–E, p < .05).
and invasiveness of ESCC cells by activating the CDCA8-mediated mTORC1 pathway in vitro for the first time, suggesting that KIF18B can be considered as a newly potential diagnostic and therapeutic target for ESCC.

Esophageal cancer is one of the lethal diseases that seriously threaten human health worldwide.1 At present, surgery, radiotherapy, and chemotherapy are recommended for the treatment of ESCC at the limited stage. Of them, chemotherapy is the main option for treating patients with ESCC who are at the terminal stage, but its efficacy has been lagging behind for many years. Besides, most of the phase III clinical studies on molecular targeted drugs for ESCC have also failed to show promising results.4,30 As a result, researchers have devoted themselves to finding targets that are more likely to produce favorable therapeutic effects and biomarkers that can better predict efficacy and prognosis.

It is well known that the uncontrolled cell cycle of cancer cells, allowing the cells to proliferate indefinitely, is a primary cause of tumor formation and spread.31 Over a decade, kinesin has become a hot topic for the study of tumor therapeutic targets, for which not only binds to microtubules, but also links the assembly of bipolar spindles in mitosis, the identification of cell division axis, and chromosome disjunction and alignment. As previously reported, kinesin inhibitor may become an important orientation of drug development for tumor growth inhibition.32 Recently, KIF18B, as family member of kinesins, has been found to highly be expressed in several cancers, which is closely correlated to the progression of cancers. For instance, in a study of Yan et al., KIF18B presents significantly higher expression in the cutaneous melanoma cells than that in normal cells and promotes tumor growth by modulating the ERK/PI3K pathway.21 Gao et al. claim that KIF18B is identified to
CDCA8 silencing reversed the promotion of KIF18B overexpression on ESCC cell migration and invasion. Both EC9706 and KYSE220 cells were transfected with KIF18B overexpression plasmids or/siCDCA8. (A–C) After transfections, the biological function of CDCA8 in cell migration was measured by wound healing assays. Original magnification, 100×. Scale bar: 50 μm. (D–F) Transwell assays were subsequently performed for detecting cell invasion. Original magnification, 200×. Scale bar: 50 μm. All experiments had gone through three repetitions. **p < .01, ***p < .001 versus Vector; *p < .05, **p < .01, ***p < .001 versus KIF18B + Vector.

CDCA8, cell division cycle associated 8; KIF18B, kinesin family member 18B
be overexpressed in osteosarcoma and facilitates cell proliferation, migration, and invasion.33 Besides, Zhong et al. also reveal the role of KIF18B that vigorously acts as a novel oncogene in lung adenocarcinoma through cell cycle regulation pathways.34 In this study, we verified the overexpression of KIF18B in ESCC cell lines and detected its function on tumor malignant phenotype by facilitating viability, cell cycle, migration and invasion of ESCC cells. Additionally, the evaluations of cell cycle- and EMT-related protein levels further confirmed our findings. Based on abovementioned findings, it is suggested that KIF18B could be considered as an oncogene in ESCC. Whereas its underlying mechanism in this cancer needed to further elucidation. Generally, the signaling process is an efficient way for cells to respond to external stimuli and ultimately trigger specific biological effects.35 From previous studies, we know that dysfunction of the mTORC1 signaling pathway can cause a variety of diseases, including obesity, type II diabetes, and cancer.36 In the research of anti-gastric cancer, Ge et al. indicate that the suppressing effect of NUSAP1 knockdown on gastric cancer is mediated by inhibiting the mTORC1 pathway.37 Another study of Li et al. reveals that the inhibition of mTORC1 and mTORC2 is a novel strategy for HNSC treatment.38 In this study, we subsequently investigated the relationship between KIF18B and the mTORC1 pathway. As a result, we observed that KIF18B activates the mTORC1 pathway by promoting phosphorylation levels of mTORC1 and p70S6K. As we know, the mTORC1 branch, as the central driver of cell progression, is found to be generally deregulated in various malignancies. In line with the above findings, it is plausible to conclude that upregulation of the effect of KIF18B on facilitating the progression of ESCC cells may be achieved by activating the mTORC1 pathway.

Currently available research studies demonstrate that CDCA8, as an encoding protein, makes an important impact upon locating chromosomal passenger complex (CPC) in centromere, correcting kinetochore bonding errors, and stabilizing the bipolar spindle.39 Analyses on CDCA8 expression shows that it is highly expressed in cancer cells and human embryonic stem cells (hESCs), but is barely expressed in normal cells.40,41 Jiao et al. report that activating CDCA8 is involved in the progression of breast cancer.42 In a recent new study, CDCA8 has been proved to regulate the progression of pancreatic ductal adenocarcinoma, which is caused by KIF18B.22 In this study, we noticed that the expression of CDCA8 is increased after ESCC cell transfected with KIF18B overexpression plasmid. Furthermore, in order to reveal the exact interaction between KIF18B and CDCA8, we knocked down CDCA8 expression in ESCC cells. Moreover, the rescue experiments signified that the effects of KIF18B overexpression on the proliferation, migration, and invasion of ESCC were reversed by CDCA8 silencing.
5 | CONCLUSION

In conclusion, this study authenticates the mechanism of KIF18B in facilitating malignant phenotype of ESCC via activating CDCA8/mTORC1 pathway. This finding may be considered as a breakthrough in the field of ESCC research and provide a novel cue for the formulation of diagnostic and treatment strategies with biomarkers.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

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