MELK Predicts Poor Prognosis and Promotes Metastasis in Esophageal Squamous Cell Carcinoma Via Activating the NF-κB Pathway

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Abstract

**Background:** Esophageal squamous cell carcinoma (ESCC) is one of the most common malignancies worldwide with a low 5-year survival rate due to the lack of effective therapeutic strategies. Accumulating evidence has indicated that Maternal embryonic leucine zipper kinase (MELK) is highly expressed in several tumors and correlates with tumor development. However, the biological effects of MELK in ESCC are still unknown.

**Methods:** We used data from ESCC patient tissue specimens and online datasets to evaluate differences in MELK expression between paired carcinoma. Two ESCC cell lines were selected and MELK was stably knocked down by small hairpin RNA (shRNA) of MELK. Cell phenotypical experiments and animal metastasis assays were performed to detect the influence of MELK knockdown *in vitro* and *in vivo*. The potential molecular mechanism of MELK-mediated ESCC metastasis was further investigated by Western blotting and Immunofluorescence staining.

**Results:** In this study, the expression of MELK in human ESCC tissues was higher than that in adjacent normal tissues and was positively correlated with the poor prognosis of patients. Reducing MELK expression resulted in growth inhibition and suppression of the invasive ability of ESCC cells *in vitro* and *in vivo*. MELK inhibition induced alterations of epithelial-to-mesenchymal transition associated proteins. Mechanistically, MELK interacted with IκB kinase (IKK) and promoted the phosphorylation of IKK, by which MELK regulated activation of the NF-κB pathway.

**Conclusions:** Collectively, our study reveals the function and mechanism of MELK in the cell metastasis of ESCC, which may be a potential therapeutic target for ESCC.

Background

Esophageal carcinoma is one of the most common malignancies worldwide with a high mortality rate [1]. China accounts for more than 50% of patients, and 90% of esophageal carcinoma includes squamous cell carcinoma (ESCC) cases [2-4]. Although the diagnosis and treatment for ESCC have improved in recent years, the overall 5-year survival rate of patients is 15% to 25% [5]. The leading cause of treatment failure and mortality among ESCC patients is tumor recurrence and metastasis [6-8]. Tumor metastasis is very complex and involves multiple steps [9]; thus, there is an urgent need to discover the underlying molecular mechanisms responsible for ESCC metastasis, as they may serve as targets to improve the treatment of ESCC.

Many protein kinases have critical roles in regulating cell growth and survival, and thus, have emerged as the most important targets for drug discovery. Maternal embryonic leucine zipper kinase (MELK) is a member of the sucrose-non-fermenting/AMP-activated protein kinase family of serine-threonine protein kinases and plays key functional roles in multiple cellular processes, including cell cycle, cell proliferation, apoptosis, and cell migration[10-13]. MELK is overexpressed in a variety of human tumors, including melanoma[14], breast cancer[15], gastric cancer[13], high-grade prostate cancer[16], and glioblastoma.
multiforme[17]. High levels of MELK expression correlate with poor prognoses in patients[16, 18, 19]. Inhibition of MELK has been shown to suppress tumor growth in vitro and pre-clinical adult cancer models[20-22]. MELK is also elevated in cancer stem cells (CSCs) and can promote CSC growth[23]. Small molecule inhibitors of MELK have anti-cancer activity in breast and other cancers[24-26], indicating that MELK might be a target for cancer therapy. Although MELK has been studied in many cancer types, few have investigated tumor progression in ESCC[27], and the function of MELK in metastasis has not been elucidated.

In this study, we found that MELK was overexpressed in human ESCC tissues and predicted a poor prognosis in patients. The high expression of MELK increased ESCC cell proliferation and invasion in vitro and stimulated ESCC metastasis in vivo. We found that MELK regulated the nuclear factor kappa B (NF-κB) pathway and mediated the epithelial-to-mesenchymal transition (EMT) process to promote tumor metastasis in ESCC. In conclusion, our study demonstrates that MELK exerts a critical regulatory role in the EMT process and consequent aggressiveness of ESCC cells by activating the NF-κB pathway.

**Methods**

**Patients and tissue samples**

A total of 84 samples of ESCC tissue (including 66 cases of tumor tissues and 18 adjacent normal esophageal tissues) were obtained from Meizhou People’s Hospital, China, between 2004 and 2008. None of the patients were treated by radiotherapy or chemotherapy before surgical operation. All samples were collected with informed consent and approved by the Research Ethics Committee of Meizhou People’s Hospital. The histopathological diagnosis was made according to the World Health Organization criteria. Tumor staging was determined based on the 6th edition of the tumor-node-metastasis (TNM) classification of the International Union Against Cancer. The characteristics of the patients are detailed in Table 1.

**Immunohistochemistry (IHC)**

The 5-μm-thick sections of tissue paraffin samples were prepared on pathological sections for immunohistochemical staining. Tissue sections were heated in citrate buffer solution (pH = 6.0) at 100 °C for 10 minutes to facilitate antigen retrieval. After tissue samples cooled down to room temperature, the sections were incubated with antibody against MELK (1:300, Proteintech, USA) for 3 hours at room temperature, and incubated with secondary antibody (Dako REAL EnVision, USA) for 30 minutes after rinsing with PBS. Immunoreacted cells were visualized using diaminobenzidine, and nuclei were counterstained with hematoxylin. The result was independently assessed by two pathologists, neither of whom knew the clinical data. The percentage of positive cells in the sections was evaluated as 0%-100%, while cases with more than 20% of tumor cells showing strong cytoplasmic staining were considered positive MELK staining. Leica Microsystems DM6000B light microscope (Leica, Wetzlar, Germany) was used for evaluation and photographic records.
Cell lines and cell culture

Human normal esophageal epithelial cell line Het-1α, human ESCC cell lines KYSE140, KYSE150, KYSE450 and KYSE510 were obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI 1640 with 10% fetal bovine serum and 1% penicillin-streptomycin in a humidified atmosphere containing 5% CO₂ at 37 °C.

RNA extraction and real-time quantitative PCR (RT-qPCR)

Total cellular RNA was obtained according to the instructions of TRIzol (Invitrogen, USA), and complementary DNA (cDNA) was prepared from 1 μg of total RNA according to the instructions of oligo dT (Takara, Japan). RT-qPCR was performed for the gene fold change by using the SYBR Premix Ex Taq™ (Takara, Japan). The mRNA levels were analyzed by the comparative 2^−ΔΔCt method after normalization to GAPDH. The primer sequences used in this research were listed as follows: MELK, (forward) 5’-TGATGATTGCGTAACAGAAC-3’, and (reverse) 5’- GAAGATAGGTAGCCGTGAG-3’; human GAPDH, (forward) 5’-ATCAATGGAAATCCCATCACCA-3’, and (reverse) 5’-GACTCCACGACGTACTCAGCG-3’.

Plasmid constructs and transfection

Corresponding shRNA (short-hairpin RNA) oligonucleotide sequences were cloned into the PLKO.1-TRC vector (Sigma-Aldrich, USA) to obtain empty vector as control and the generation of lentiviral vectors encoding shRNA targeting MELK(The shRNA sequences including shRNA#1, TRCN0000001643; shRNA#2, TRCN0000001644; shRNA#3, TRCN0000001645 referenced from https://www.sigmainformatics.com). The cells were transfected by purified lentiviruses. After selected by 2.5 mM puromycin (Sigma-Aldrich, USA) for 3 days, the cells with stable knockdown of MELK were picked up and expanded. Then, Western blotting was performed to identify the knockdown of MELK. For overexpression of IKKβ, MELK depleted ESCC cells were transfected with IKKβ expression plasmid (pcDNA3.1-IKKβ) or the empty vector (pcDNA 3.1-EV) as control. After selected by 400 ug/ml G418 (MedChemExpress, USA) for 2 weeks, the cells with stable overexpression of IKKβ were picked up and expanded.

Proliferation assays with cell counting kit-8 (CCK-8)

Cell Counting Kit-8 (CCK-8) assays, according to the manufacturer’s instruction, were performed to detect cell proliferation. Cells were seeded into 96-well plates with a density of 1,000 cells per well in 100 μl cell medium. Each group was tested every 24 hours. The plates were placed in a 37 °C incubator for 2 hours after adding 10 μl of CCK8 working solution (Bimake, USA), and then the absorbance was measured at 450 nm using a microplate reader. The average values of the five replicates were calculated and used to draw the growth curve. The above experiment was repeatedly performed three times. The OD₄₅₀ value was statistically evaluated by one-way ANOVA analysis using SPSS 19.0 statistical software.

Colony formation assay
Colony formation assays were performed to detect cell proliferation. Cells were inoculated into 6-well plates with a concentration of 100 cells per well. The plates were placed in a cell incubator at 37 °C for 2 weeks, and the medium with 10% FBS was renewed every 4 days. When visible colonies have formed, each well was fixed with 1 ml of 4% paraformaldehyde for 30 minutes and stained with 1 ml of 0.1% crystal violet (Beyotime, China) for 30 minutes. The number of visible colonies of three replicates was counted after the plates dried up. The experiment was independently performed three times.

Transwell Assays

Transwell assays were performed to detect cell vertical migration and invasion. 5×10⁴ cells in 150 μl serum-free medium were seeded in the upper part of the transwell chamber after pre-treated with serum-free medium for 12h. The upper chambers had been pre-coated with 50 μl of 2.5 mg/ml solution of matrigel (Corning, USA) for the invasion assays or uncoated for the migration assays. The lower chambers were filled with 600 μl RIPA 1640 containing 10% FBS. After 24 hours (migration experiment) or 36 hours (invasion experiment), the cells on the upper surface of chambers membranes were wiped off. Then, the membranes were fixed with 4% polymethanol for 30 minutes and stained with 0.5% crystal violet for 30 minutes. Five fields randomly selected under the microscope were photographed to count the cells on the lower surface of the filter at a magnification of ×100. This experiment was performed in triplicate independently.

Wound healing assays

Wound healing assays were performed to detect cell lateral migration. Cells were seeded in a 6-well plate and exposed to serum-free RPMI 1640 for 12 hours to inhibit cell proliferation. After the wound was induced, the cells were washed twice with PBS and cultured in a serum-free medium. Photos of migration were taken at 0 h, 16 h, and 20 h after inducing under a microscope. The assays were repeatedly performed three times.

Western Blotting

Western blotting analysis was performed to detect the expression level of related protein. The whole cell lysates were prepared in SDS buffer with 1% protease inhibitor cocktail and 1% PMSF. The nuclear proteins were obtained by using Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, China) according to the instructions. The concentration of protein samples was quantified by using BCA Protein Assays Kit (Beyotime, China). 50 μg of proteins were separated by 10% SDS-PAGE and then transferred onto PVDF membranes. Incubated with TBST buffer including 5% dried skimmed milk at room temperature for 1 hour, the corresponding PVDF membranes were incubated with primary antibody at 4°C overnight. Then, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 hour. Finally, protein bands were detected using enhanced chemiluminescence (ECL) detection reagent (Millipore, USA) according to the instructions. The grayscale value of the immunoreactive bands was measured by Image J (National Institutes of Health, USA). Antibodies used in this study are listed as follows: Antibodies against ZO-1 (Cat. #8193), E-Cadherin (Cat.
N-Cadherin (Cat. #13116), MMP-2 (Cat. #40994), MMP-9 (Cat. #13667), ZEB2 (Cat. #97885), Slug (Cat. #9585), Snail (Cat. #3879), p-IKKα/β (Ser176/180, Cat. #2697) were purchased from Cell Signaling Technology (Danvers, USA). Antibodies against GAPDH (Cat. #60004-1-Ig), MELK (Cat. #11403-1-AP), p65 (Cat. #10745-1-AP) were obtained from Proteintech (Rosemont, USA). Antibodies against NF-κB1 (Cat. #BM3946), NF-κB2 (Cat. #BA1298) were purchased from Boster Biological Technology (California, USA). Antibodies against p-p65 (Ser536, Cat. #WL02169), p-IκB-α (Ser32/36, Cat. #WL02495) were purchased from Wanlei Biological Technology (Shenyang, China). Antibody against p62 (SQSTM1) (Cat. #PM045) were obtained from MBL Biological Technology (Beijing, China).

**Immunofluorescence staining**

Cells were singly planted on cell slides and fixed with 4% paraformaldehyde for 20 minutes. Washed thrice with PBS for 5 minutes each time, cells were infiltrated with 0.3% Triton X-100 (Sigma-Aldrich, USA) in PBS and fixed with 10% goat serum in PBS. Cells were incubated with primary antibody overnight at 4°C and then were incubated with Alexa Fluor 488/647-conjugated secondary antibodies (1:500; Cell Signaling Technology, USA) for 1 hour. Washed three times with PBS for 10 minutes each time, cells were counterstained with DAPI for 15 minutes at room temperature. Fluorescence images were captured by Zeiss LSM 780 or LSM 800 laser scanning microscope (Carl Zeiss, Germany). Antibodies used in this study are listed as follows: MELK (1:100, proteintech, Cat. #11403-1-AP, USA), Slug (1:100, Cell Signaling Technology, Cat. #9585, USA), NF-κB1 (1:25, Boster Biological Technology, Cat. #BM3946, USA), NF-κB2 (1:50, Boster Biological Technology, Cat. #BA1298, USA).

**Co-immunoprecipitation (Co-IP) Assays**

Protein lysate of KYSE510 cells was collected using IP lysis buffer and mixed with the indicated antibody or negative control IgG overnight at 4 °C. Mixed with 20 μl of beads for 4 hours at 4°C, the binding complex was obtained by using a magnetic stand. After washed thrice with IP buffer, the complex was boiled with SDS buffer to gain bounding protein samples, which were used for western blotting analysis. Antibodies used in this study are listed as follows: MELK (1:50, proteintech, Cat. #11403-1-AP, USA), IKKα/β (1:50, Wanleibio, Cat. #WL01900, China), IgG (1:50, Beyotime, Cat. #A7016, China).

**Bioinformatics Analysis**

Two human microarray datasets, namely, GSE20347 and GSE23400, were obtained from the public Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo). The mRNA sequencing data of patients with ESCC were downloaded from The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/). Gene expression of MELK in the GEO database and TCGA ESCC database were assessed using GraphPad Prism 7 software. Gene set enrichment analysis (GSEA) was performed by generating the Gene MatriX file (.gmx) by using published signatures for the RICKMAN METASTASIS[28], the SARRIO EPITHELIAL MESENCHYMAL TRANSITION[29], and GO biological process gene sets from the Molecular Signatures Database (MSigDB). The gene cluster text file (.gct) was
generated from the TCGA ESCC database. The number of permutations for GSEA was set to 1,000, and we used the TCGA gene list as the chip platform.

**Experimental in-vivo metastasis assays**

Athymic male nude mice were purchased from the Animal Center of the Chinese Academy of Sciences (Shanghai, China) and raised in a laminar flow cabinet free of specific pathogens. KYSE150 cells stably transfected with shNC or shMELK were collected and re-suspended in PBS at a concentration of $1 \times 10^8$ cells/ml. Prepared cells were injected into the tail veins of 14 mice (100 μl per mouse), which were sacrificed 5 weeks after injection. The lungs and livers of mice were removed and photographed. Then, the visible tumors on the surface of lung tissue were counted. The experiment was strictly carried out following the Guide for the Care and the Use of Laboratory Animals of the National Institutes of Health. It was approved by the Committee on the Ethics of Animal.

**Statistical Analysis**

All data were analyzed with SPSS 19.0 software (IBM, USA). The association between MELK expression and clinicopathological feathers of ESCC patients was analyzed by the chi-squared test. The Kaplan-Meier method and log-rank tests were used to compare the overall survival. The numerical data are reported as the mean ± standard deviation (SD). The difference between two groups was analyzed by a two-tailed Student’s t-test. Three or more groups were analyzed by a one-way ANOVA with Tukey’s post hoc test. The difference $P$ values of $<0.05$ were considered statistically significant.

**Results**

**MELK is highly expressed in human ESCC tissues and correlates with poor overall survival of ESCC patients**

MELK is highly expressed in cancer and plays key roles in tumor progression in some cancer types; however, the role of MELK in ESCC is still unknown. To assess the roles of MELK in ESCC, we first measured the expression levels of MELK in 84 ESCC tissues and 18 adjacent normal esophageal tissues by immunohistochemistry staining. The MELK protein was found to be localized in the cytoplasm (Figure 1A). The positive rates of MELK protein detection were 5.6% (1/18) in non-tumor tissues and 52.4% (44/84) in tumor tissues, and the expression of MELK was significantly upregulated in ESCC compared with normal tissues ($P = 0.000$, Figure 1B). We also analyzed the correlations between the expression level of MELK and clinicopathological characteristics of patients, although the results showed that the higher MELK expression was not significantly correlated with characteristics, including age, histological grade, and tumor stage ($P > 0.05$; Table 1). By using Kaplan-Meier analysis, we found that high MELK expression suggested the poor overall survival of ESCC patients ($P < 0.05$; Figure 1C). Furthermore, we queried the Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA) databases to analyze MELK levels in ESCC patients, and the increased expression of MELK was also observed in ESCC (Figure
1D-F). These results indicate that the expression of MELK protein is elevated in ESCC and predicts a poor prognosis in patients.

**MELK inhibition reduces proliferation and colony formation in ESCC cells**

Our results showed that MELK was more highly expressed in ESCC and positively correlated with the poor survival of patients. To determine the biological function of MELK in ESCC, we first analyzed the protein level of MELK in ESCC cell lines, and then selected KYSE150 and KYSE510 cell lines, which had higher expression levels of MELK, to be used for further study (Figure 2A). We generated stable knockdown of MELK by transfecting KYSE150 and KYSE510 cells with small hairpin RNA (shRNA) of MELK. Following transfection and selection with puromycin, the expression of MELK in KYSE150 and KYSE510 cells was significantly decreased (Figure 2B). Then we investigated the effect of MELK on the proliferation of ESCC cells using Cell Counting Kit-8 (CCK-8) assays. The results showed that knockdown of MELK significantly reduced the growth of KYSE150 and KYSE510 cells compared with the control cells (Figure 2C). Colony formation assays demonstrated that knockdown of MELK efficiently decreased the ability of ESCC cells to proliferate (Figure 2D). These results suggest that MELK regulates the proliferative ability of ESCC cells.

**MELK inhibition decreases the migration and invasion of ESCC cells**

Next, we examined the cell migration and invasive ability with transwell migration, transwell invasion, and wound healing assays. The number of migrated and invaded cells in MELK knockdown KYSE150 and KYSE510 cells was significantly decreased compared with the control cells (Figure 3A-B). In a wound healing assay, the control cells obviously closed the wound 16 hours after scratching, but MELK knockdown cells were unable to heal the wound, and the wound areas of the experimental group and controls were significantly different. MELK knockdown significantly inhibited cell migration (Figure 3C). To further ascertain whether MELK accelerates the motility capacity of ESCC cells, we used Gene Set Enrichment Analysis (GSEA) to determine the degree of MELK expression with metastasis-related signatures. Our results showed that ESCC with high MELK expression had significant enrichment with an aggressive signature. To further confirm this observation, we correlated MELK expression with metastasis-related markers and found that GSEA highlighted the positive correlation of increased MELK expression with metastasis and the negative correlation of increased MELK expression with adherens junction and tight junction (Figure 3D). Taken together, these findings indicate that high MELK expression may be associated with the highly infiltrative and aggressive characteristics of ESCC cells.

**MELK facilitates the EMT in ESCC cells**

EMT is an early event in tumor metastasis, and tumor cells show enhanced migration and invasion ability during the EMT. Therefore, we examined the expression of markers associated with the EMT by Western blotting. MELK inhibition upregulated the expression of epithelial-related proteins, including E-cadherin and zonula occludens-1 (ZO-1), and downregulated mesenchymal-related protein N-cadherin in both KYSE150 and KYSE510 cells (Figure 4A). Moreover, EMT-inducing transcription factors (EMT-TFs), zinc
finger E-box-binding homeobox 2 (ZEB2), Slug and Snail, were also inhibited after MELK knockdown (Figure 4B). In particular, the protein expression of Slug was shown to be obviously reduced by immunofluorescence staining (Figure 4C). In addition, MELK knockdown decreased matrix metalloproteinase 9 (MMP9) and MMP2 protein levels, which are involved in facilitating cell metastasis by extracellular matrix (ECM) degradation (Figure 4A). Furthermore, GSEA highlighted the positive correlation of increased MELK expression with the EMT signature (Figure 4D). Thus, our results showed that MELK may promote cell migration and invasion by promoting EMT in ESCC cells.

**MELK positively regulates NF-κB signaling**

To decipher the mechanisms by which MELK regulates EMT, we assessed the involvement of the NF-κB pathway. Aberrant NF-κB pathway activation is a significant contributor to the EMT[30, 31]. The NF-κB pathway can directly and indirectly affect Snail, Slug, and ZEB1 expression to facilitate the EMT. We examined the expression/phosphorylation of proteins involved in NF-κB signaling by Western blotting. The results showed that MELK inhibition significantly downregulated the expression of main NF-κB subunits, including NF-κB p50, NF-κB p52, NF-κB p65, and phosphorylated NF-κB (p-NF-κB) p65 (Figure 5A). We also examined the nuclear translocation of NF-κB p65, NF-κB p50, and NF-κB p52, and MELK inhibition led to decreased translocation of NF-κB p65, NF-κB p50, and NF-κB p52 to the nucleus (Figure 5B-C), which suggested downregulation of this signaling pathway. Additionally, Slug, the downstream target of the NF-κB pathway was evaluated, and its expression was shown to be reduced in the nucleus (Figure 5B). These results suggested that MELK knockdown inhibited activation of the NF-κB pathway. NF-κB complex is usually inactive and located in the cytoplasm while bound to IκB inhibitor proteins. When IκB protein is phosphorylated by the IκB kinase (IKK) complex, which leads to IκB ubiquitination and subsequent degradation[32], NF-κB subunits may translocate to the nucleus and activate downstream gene transcription[31, 33]. Thus, we examined the expression levels of NF-κB upstream kinases; the results showed that MELK inhibition reduced the protein levels of p-IκB, p-IKK, and p62 by which the activation of NF-κB was decreased (Figure 5D). Furthermore, we performed co-immunoprecipitation in ESCC cells to detect the interaction between MELK and the upstream kinases of the NF-κB signaling and found that MELK could bind to IKK and regulated phosphorylation of IKK (Figure 5E). Hence, our results suggested that MELK may activate the canonical NF-κB signaling by interacting with IKK.

**Overexpression of IKKβ rescues NF-κB signaling and the migration of ESCC cells after MELK inhibition**

Because IKKβ acts downstream of MELK, we investigated whether ectopic expression of IKKβ could rescue the inhibition of NF-κB signaling caused by MELK inhibition. IKKβ-overexpressing plasmid was stably transduced into MELK depleted ESCC cells, and then the expression of IKKβ was detected by Western blotting. As shown in Figure 6A, the protein levels of p-IKKβ, IKKβ and its downstream targets including p-IκBα, p-p65 and NF-κB p50 were drastically upregulated by IKKβ in ESCC cells with MELK depletion. A previous study reported that MELK promoted tumor growth and metastasis via stimulating FOXM1 signaling in ESCC[27]. In view of the reciprocal regulation between NK-κB and FOXM1 [34], we
also assessed the expression of FOXM1 in ESCC cells with MELK knockdown and IKKβ overexpression. As shown in Figure 6A, MELK knockdown inhibited FOXM1 expression, which is rescued by IKKβ overexpression. Immunoblotting analysis also showed that ectopic expression of IKKβ remarkably restored Slug, E-cadherin, N-cadherin, ZO-1, MMP-2 and MMP-9 expression in MELK depleted ESCC cells (Figure 6B). Moreover, overexpression of IKKβ in MELK depleted ESCC cells also attenuated the inhibitory effects on the migration in both transwell and wound healing assay (Figure 6C-D). In contrast, expression of an empty vector did not rescue the migration of ESCC cells (Figure 6C-D). Altogether, these results demonstrate that attenuation of NF-κB signaling is partly responsible for blocking ESCC migration inhibition after MELK inhibition.

**MELK inhibitor blocks the EMT by NF-κB pathway**

To further confirm whether MELK inhibition, but not an off-target effect, would reduce the activation of the NF-κB pathway, we treated ESCC cell lines with the MELK inhibitor OTSSP167[19, 35, 36]. OTSSP167 treatment significantly inhibited ESCC cell migration (P < 0.05; Figure 7A). OTSSP167 reduced the protein levels of p-IKK, p-IκB, NF-κB p50, NF-κB p52, NF-κB p65, and p-NF-κB p65 and the downstream protein Slug (Figure 7B-C). Thus, MELK inhibition was confirmed to block the EMT by decreasing the activation of NF-κB signaling in ESCC cells.

**Knockdown of MELK inhibits metastatic capacities of ESCC cells in vivo**

To determine whether MELK regulates ESCC cell migration and invasion potential in vivo, KYSE150-shMELK and control KYSE150 vector cells were injected into the tail vein of nude mice (seven mice per group). At 5 weeks after cell injection, mice were killed, and metastatic tumors formed in the lung and liver were examined. The results showed that no tumor nodule was formed in the liver of mice. However, metastatic tumor nodules were frequently observed in the lung of mice, and knockdown of MELK significantly decreased the numbers and volume of metastatic nodules in the lung (P < 0.05; Figure 8A-C). These results showed that MELK inhibition significantly reduced metastasis in ESCC.

**Discussion**

Recent studies have shown that MELK plays an important role in tumor progression of many kinds of tumors[13, 16, 19, 37]. However, little is known about the function and related mechanisms of MELK in ESCC[27]. In this study, we showed that MELK expression was upregulated in ESCC tissues and was important for the acquisition of an aggressive and poor prognostic phenotype. We also demonstrated that MELK inhibition decreased cell proliferation, migration, and invasion in ESCC cells both in vitro and in vivo. Furthermore, we observed that MELK induced EMT and promoted cell migration and invasion via the NF-κB pathway (Fig.8D).

ESCC is one of the most common malignant tumors, and the vast majority of tumor-related deaths are the result of tumor metastasis, but the precise molecular mechanisms that drive this metastasis process are largely unknown. Therefore, it is important to understand the molecular events governing tumor
proliferation and metastasis to develop novel therapeutic targets in ESCC. Many studies have shown that MELK is highly expressed in several tumors, and its expression is correlated with tumor grade and prognosis[38-40]. In this study, our results showed that MELK expression was significantly upregulated in ESCC compared with normal esophagus epithelium, which is consistent with the previous studies in other types of tumors[19, 25]. Survival analysis showed that higher MELK expression predicted a poor prognosis in patients with ESCC. To further confirm our findings, large sample data from TCGA and GEO database confirmed the result of MELK expression in our tissue samples. However, the role of MELK in human ESCC remains poorly studied.

As a serine/threonine protein kinase, MELK was reported to regulate the cell cycle, stem cell renewal, and apoptosis[25, 41, 42]. MELK was also identified to be contributing to tumor metastasis and recurrence in breast and gastric cancers[13, 43]. In the current study, a series of assays were employed to investigate the role of MELK in regulating the characteristic aggressive phenotype of MELK. We established knockdown expression of MELK ESCC cells \textit{in vitro} due to the high expression in ESCC cell lines and investigated the cell growth, colony formation, and invasion of these cells. Most previous studies on the function of MELK in tumors have focused on cell growth and apoptosis. The results of this study suggested that MELK inhibition decreased cell proliferation and colony formation in ESCC cells, consistent with previous studies[19, 26, 44]. However, the cell cycle and apoptosis were not found to be significantly influenced by MELK inhibition in ESCC cells. Of note, the cell migration and invasion ability were inhibited in MELK knockdown ESCC cells. The metastasis of ESCC cells was also reduced by MELK inhibition \textit{in vivo}. Taken together, our results demonstrate that MELK is an important oncogenic factor and plays critical roles in the cell growth and metastasis of ESCC.

The migration and invasion of cancer cells involve multiple changes in tumor cells[45, 46]. EMT is considered one of the major mechanisms involved in solid tumor metastasis[47]. It has been reported that EMT progression is accompanied by several crucial changes of epithelial cells, including loss of epithelial adherence, tight junction proteins, loss of cell polarity, and acquisition of a mesenchymal phenotype, leading to invasive and migratory behavior[9, 29]. In this study, we examined the expression levels of EMT-related proteins and found that the epithelial markers E-cadherin and ZO-1 were upregulated, while mesenchymal markers N-cadherin, ZEB1, Snail and Slug downregulated by MELK inhibition in ESCC cells. In addition, MMPs, which are capable of degrading the ECM, were upregulated. Furthermore, GESA analysis confirmed the correlation between MELK and aggressive characteristics in ESCC. Collectively, these results reveal that MELK is an important factor for the support of EMT and aggressiveness in ESCC cells.

To date, the precise molecular mechanisms of MELK that promote ESCC cell EMT and aggressive potential remain unknown. MELK reportedly regulates tumor progression by several signaling pathways[19, 48, 49]. The NF-κB pathway is a major tumor promotion and EMT-related pathway[30, 50, 51]. A previous study showed that MELK regulates the NF-κB pathway by phosphorylating SQSTM1/p62 and promoting melanoma growth[14]. Our results showed that MELK inhibition decreased the expression of NF-κB transcriptional targets, and reduced translocation to the nucleus of p65, p50, and p52. The
expression of p62 was also decreased in MELK knockdown cells, which was consistent with the previous study[14]. The activation of the NF-κB complex relies on IkB protein being phosphorylated by the IKK complex, which leads to IkB ubiquitination and subsequent degradation. We found that the phosphorylation of IkB and IKK were decreased in MELK knockdown ESCC cells. Our findings were also confirmed by treatment of ESCC cells with the MELK inhibitor OTSSP167[25, 26, 36]. In addition, co-immunoprecipitation experiments showed that MELK interacted with IKK. The inhibitory effect of MELK knockdown on migration ability and NF-κB signaling could be rescued by overexpression of IKKβ dramatically. Therefore, we propose that MELK activates the NF-κB pathway by phosphorylating IKKβ. A previous study claimed that MELK enhanced tumorigenesis, migration, invasion and metastasis of ESCC cells via activation of FOXM1 signaling pathway[27]. FOXM1 promoter region contains a functional NF-κB element and is transcriptionally activated upon NF-κB binding in chronic myelogenous leukemia cells[52]. There are also FOX binding motifs within the FOXM1 promoter, which activity is markedly induced after overexpressing p65[34]. In the present study, ectopic expression of IKKβ remarkably rescued FOXM1 expression in MELK-silenced ESCC cells, which indicates that FOXM1 is a downstream effector of IKKβ function. Collectively, these results identify MELK as a regulator of the NF-κB pathway and show that MELK at least partly promotes ESCC metastasis by activating this pathway.

Conclusion

In summary, the results of our study showed that MELK regulated the activation of the NF-κB pathway via its inhibitor IKK. In addition, MELK promoted tumor metastasis in ESCC and predicted a poor prognosis in patients. Therefore, MELK may be a potential focus of future therapeutic options for ESCC.

Abbreviations

ESCC: Esophageal squamous cell carcinoma; MELK: Maternal embryonic leucine zipper kinase; NF-κB: Nuclear factor kappa B; shRNA: Small hairpin RNA; IKK: IkB kinase; CSCs: Cancer stem cells; EMT: Epithelial-to-mesenchymal transition; TNM: Tumor-node-metastasis; IHC: Immunohistochemistry; CCK-8: Cell Counting Kit-8; Co-IP: Co-immunoprecipitation; GEO: Gene Expression Omnibus; TCGA: The Cancer Genome Atlas; GSEA: Gene set enrichment analysis; ZO-1: Zonula occludens-1; EMT-TFs: EMT-inducing transcription factors; ZEB2: Zinc finger E-box-binding homeobox 2; MMP9: Matrix metalloproteinase 9; ECM: Extracellular matrix.

Declarations

Ethics approval and consent to participate

This study was authorized by the Ethical Committee of Meizhou People's Hospital. All procedures were performed according to the Declaration of Helsinki. Animal experiments were conducted based on the minimized animal number and the least pains according to the Guide for the Care and Use of Laboratory Animals formulated by the National Institutes of Health.
Consent for publication

Not applicable.

Availability of data and materials

The data that support this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare no conflict of interest.

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Author Contributions

Lihui Wang designed the study and revised the manuscript. Jiecheng Ye, Wanying Deng, Ying Zhong, Hui Liu, Baoyin Guo, Zixi Qin and Peiwen Li completed the experiments. Jiecheng Ye and Wanying Deng performed data analysis and drafted the manuscript. All the authors read and approved the final manuscript.

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**Tables**

Table 1. Relationship between MELK expression and clinical pathological features in 84 patients with ESCC
| Variables               | N | MELK Expression | P     |
|------------------------|---|----------------|-------|
|                        |   | Low | High |
| Sex                    |   |     |      |
| Male                   | 67| 28  | 39   |
| Female                 | 17| 12  | 5    |
| Age (years)            |   |     |      |
| ≤55                    | 42| 19  | 23   |
| ≥55                    | 42| 21  | 21   |
| Tumor Size (cm)        |   |     |      |
| ≤5.0                   | 44| 19  | 25   |
| ≥5.0                   | 40| 21  | 19   |
| Histological Grade     |   |     |      |
| 1                       | 12| 5   | 7    |
| 2—3                    | 72| 35  | 37   |
| T stage                |   |     |      |
| T1—T2                  | 24| 13  | 11   |
| T3—T4                  | 60| 27  | 33   |
| N stage                |   |     |      |
| N0                     | 50| 25  | 25   |
| N1                     | 34| 15  | 19   |
| Tumor Stage            |   |     |      |
| 1—2                    | 54| 28  | 26   |
| 3—4                    | 30| 12  | 18   |

*Statistically significant. Abbreviation: N, number of cases; P, p value.

**Figures**
MELK expression was elevated and correlated with poor prognosis in ESCC. (A) Representative MELK immunohistochemistry staining of ESCC and normal esophageal tissue (magnification, 50× and 200×). Scale bars, 50 μm. (B) The histogram illustrates the percentage of MELK immunostaining in ESCC and normal esophageal tissue. ESCC tissues showed significantly higher MELK expression levels than normal esophageal tissue (chi-square test, P=0.000). (C) Kaplan-Meier curves according to MELK expression status in ESCC. Patients with positive MELK expression have a shorter overall survival time than patients with negative MELK expression (log-rank test, P=0.045). (D-E) The relative expression of MELK in ESCC tissues and paired normal tissues were compared using GEO datasets, series GSE20347 and GSE23400. (F) The relative expression of MELK in ESCC tissues and normal tissues was compared using TCGA data. *P< 0.05, **P< 0.01, ***P< 0.001.
Figure 2

Knockdown of MELK inhibited cell growth in ESCC. (A) Expression levels of MELK in ESCC cell lines. (B) The knockdown efficiency of MELK shRNA was confirmed by Western blotting in KYSE150 and KYSE510 cells. (C) Cell Counting Kit-8 assays were performed to detect cell proliferation. (D) Colony formation assays were performed to detect cell proliferation. The histograms illustrate the number of colonies.
counted. Data are presented as mean ± SD of 3 independent experiments. *P< 0.05, **P< 0.01, ***P< 0.001.

**Figure 3**

Knockdown of MELK inhibited cell invasion and migration in ESCC. (A-B) Representative transwell migration (A) and invasion (B) images and statistics of the number of counted KYSE150 and KYSE510 cells with MELK knockdown. Scale bars, 100 μm. (C) Micrographs and histograms of the scratch wound-
healing assay of ESCC cells with MELK knockdown. Scale bars, 500 μm. (D) GSEA was performed using the signatures for the RICKMAN METASTASIS and GO biological process gene sets. Patients were separated by high or low MELK mRNA expression. NES normalized enrichment score. Data are presented as mean ± SD of 3 independent experiments. *P< 0.05, **P< 0.01, ***P< 0.001.

Figure 4

MELK promoted ESCC migration and invasion by regulating the expression of EMT-associated proteins. (A-B) Western blotting analysis of EMT-associated proteins in ESCC cells with MELK knockdown. (C) Immunofluorescence staining of MELK and Slug protein in ESCC cells. Scale bars, 50 μm. (D) GSEA was performed using the signatures for the SARRIO EPITHELIAL MESENCHYMAL TRANSITION. Data are presented as mean ± SD of 3 independent experiments.
Figure 5
MELK promoted ESCC migration and invasion by activating NF-κB pathway. (A&D) Western blotting analysis of NF-κB pathway-associated proteins in ESCC cells with MELK knockdown. (B) Western blotting analysis of NF-κB pathway-associated proteins in nuclear of ESCC cells with MELK knockdown. (C) Immunofluorescence staining of NF-κB pathway-associated proteins in ESCC cells. Scale bars, 50 μm. (E)
Co-immunoprecipitation assays were performed using either MELK or, as a control, IgG antibodies. Data are presented as mean ± SD of 3 independent experiments.

**Figure 6**

Overexpression of IKKβ rescued the migration ability of MELK depleted ESCC cell lines. (A) Western blotting analysis of NF-κB pathway-associated proteins in ESCC cells with IKKβ overexpression. (B) Western blotting analysis of EMT-associated proteins in ESCC cells with IKKβ overexpression. (C)
Representative transwell migration images and statistics of the number of counted ESCC. Scale bars: 100 μm. (D) Representative wound-healing assays of migration images and statistics of the percentage of ESCC. Scale bars: 200 μm. Data are presented as mean ± SD of 3 independent experiments. *P< 0.05, **P< 0.01, ***P< 0.001.

Figure 7

OTSSP167 reduced ESCC migration and invasion by inhibiting NF-κB pathway. (A) Wound healing assays of migration in ESCC treated with OTSSP167 for 32h. Scale bars, 100 μm. (B-C) Western blotting analysis of NF-κB pathway-associated proteins in ESCC cells treated with OTSSP167 for 24h. Data are presented as mean ± SD of 3 independent experiments. *P< 0.05, **P< 0.01, ***P< 0.001.

Figure 8

Knockdown of MELK inhibited the metastasis of ESCC cells in vivo. (A) KYSE150 cells with MELK knockdown were intravenously injected into nude mice (n = 7 per group) through the tail vein. Representative images of excised lungs after 5 weeks injection are shown (arrows indicate the metastatic foci). (B) The number of tumor nodules on lung surfaces. (C) Lung metastases in each mouse were
stained with H&E. Arrows indicate the metastatic colonization of tumor cells in the lung tissues. Scale bars, 1 mm (left), 200 µm (right). (D) Schematic illustrates the role of MELK in stimulating metastasis of ESCC. Scale bars, 200 µm. *P< 0.05, **P< 0.01, ***P< 0.001.