Maternal embryonic leucine zipper kinase: A novel biomarker and a potential therapeutic target in lung adenocarcinoma

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Abstract. Maternal embryonic leucine zipper kinase (MELK), is an adenosine monophosphate-activated protein kinase-related kinase that serves important roles in tumourigenesis in multiple malignant tumours. However, to the best of our knowledge, the effect of MELK in lung adenocarcinoma (LUAD) has not been elucidated. The present study aimed to explore the clinical significance of MELK in the prognosis of LUAD. Data from Oncomine, Gene Expression Profiling Interactive Analysis (GEPIA) and The Cancer Genome Atlas (TCGA) were selected to predict the differential mRNA expression levels of MELK mRNA in LUAD and normal tissues. Subsequently, LUAD and adjacent normal tissue samples were collected from 75 patients with the disease, and immunohistochemistry was used to detect the protein expression of MELK. In addition, the Kaplan-Meier Plotter database, GEPIA and TCGA were used to verify the effect of MELK expression on clinical prognosis in patients with LUAD. MELK was significantly upregulated in LUAD tissues compared with that in normal tissues based on Oncomine, GEPIA and TCGA data (P<0.05). In addition, the results from immunohistochemistry demonstrated that the MELK protein level in LUAD tissues was significantly higher compared with that in matched normal tissues (P<0.05). Prognostic analysis performed using the Kaplan-Meier plotter, GEPIA and TCGA suggested that the expression of MELK was negatively associated with the overall survival time of patients with LUAD (P<0.05). In conclusion, MELK was highly expressed in LUAD based on bioinformatics and immunohistochemistry analysis, and increased expression of MELK was associated with a poor patient prognosis. MELK may serve as a potential diagnostic marker and therapeutic target for LUAD.

Introduction

Lung cancer is the most common malignancy and the leading cause of death among all types of cancer worldwide, with 11.6% of the total cancer and 18.4% of the total cancer deaths according to the Global Cancer Statistics 2018. Lung cancer contains multiple subtypes, such as small cell lung cancer (SCLC), squamous cell carcinoma and lung adenocarcinoma (LUAD). LUAD is the most common type of lung cancer (1,2). In recent decades, an increased understanding of the underlying molecular mechanisms of LUAD has been obtained, and notable progress has been made in analysis of LUAD prognosis, especially in terms of tumour markers (1). For example, in a previous study, epidermal growth factor receptor (EGFR) expression was high in 62% of patients with non-small cell lung cancer (NSCLC), squamous cell carcinoma and lung adenocarcinoma (LUAD). LUAD is the most common type of lung cancer (1,2). In recent decades, an increased understanding of the underlying molecular mechanisms of LUAD has been obtained, and notable progress has been made in analysis of LUAD prognosis, especially in terms of tumour markers (1). For example, in a previous study, epidermal growth factor receptor (EGFR) expression was high in 62% of patients with non-small cell lung cancer (NSCLC), and when treated with inhibitors of EGFR, these patients had an improved prognosis compared with patients without EGFR upregulation (3). Therefore, targeted therapy is becoming increasingly popular, and new and more efficient diagnostic and therapeutic targets in LUAD are urgently needed.

Growing evidence has confirmed that the expression of certain genes to which the cancer has become ‘addicted’ or ‘dependent on’ is necessary for tumour growth (4,5). Silencing these genes or inhibiting the activity of the proteins they encode may promote cancer apoptosis (4,5). Maternal embryonic leucine zipper kinase (MELK), an adenosine
monophosphate-activated protein kinase-related kinase, is upregulated in multiple malignant tumours, including breast cancer, melanoma, glioblastoma, hepatocellular carcinoma, acute leukaemia and ovarian cancer (6-11). In addition, some cancer types have been reported to depend on the upregulation of MELK, such as breast cancer, glioma and melanoma, and MELK serves as an oncogenic driver gene inhibiting increased cell apoptosis and suppressed growth of tumours (4). OTSSP167, a selective small molecule inhibitor of MELK, is currently being evaluated in phase I/II clinical research in patients with breast cancer, glioblastoma and acute leukaemia (8,11-13). Notably, bioinformatics screening studies conducted using data from Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA), among others, have demonstrated that MELK is one of the most frequently identified hub genes in lung cancer, and high expression of MELK is associated with worse overall survival (OS) time compared with low expression of MELK in patients with NSCLC (1,14,15). This suggests that MELK may be a key biomarker for diagnosis and therapy; however, further clinical data, especially from clinical samples, are needed to increase the accuracy and credibility of such findings.

Therefore, in the present study, the differential expression of MELK in patients with LUAD and its association with prognosis were assessed. Oncomine, Gene Expression Profiling Interactive Analysis (GEPIA) and TCGA data were used to determine the levels of MELK mRNA in malignant tumour and normal lung tissues. In addition, immunohistochemical staining was used to detect the expression of MELK protein in LUAD and adjacent normal tissue. Kaplan-Meier plotter, GEPIA and TCGA survival analyses were conducted to compare the prognostic outcomes in patients with LUAD with different levels of MELK.

Materials and methods

Bioinformatics mining methods. Data from the Oncomine (https://www.oncomine.org) (16) and TCGA (https://tcga-data.nci.nih.gov/tcga/) databases (17,18), and GEPIA (http://geopia.cancer-pku.cn) (18,19), were used to estimate the expression of MELK in LUAD and adjacent normal tissues (16-19). The search parameters in Oncomine were as follows: Analysis type, cancer vs. normal; data source, public; cancer type, selected specific cancer type; sample type, clinical specimen; and data type, mRNA. Fold-change (FC) ≥2, P<0.05 and a gene rank in the top 10% were set as the thresholds for selecting the datasets. The results of differential expression analyses, including subgroup, are also from TCGA and GEPIA. FC ≥2 and P<0.05 were set as the thresholds of gene upregulation. Among them, Oncomine and TCGA databases provide the number of samples in different groups; however, GEPIA does not provide the number of samples in different groups. Kaplan-Meier plotter from dataset ID 204825 (http://kmplot.com/analysis), GEPIA and TCGA were used to analyse the prognosis of patients with differential expression of MELK (20). Briefly, the patient samples were divided into 2 groups according to transcripts per million (TPM) value. The data with TPM greater than upper quartile was assigned to a high expression group and the others with TPM below upper quartile belonged to low/medium expression group. Then the gene expression data were put into R software to obtain the survival plots, in which the number-at-risk was shown below the main plot.

Patients. A total of 75 cancer tissue and 35 normal adjacent tissue samples were obtained from patients diagnosed with LUAD who underwent surgical resection were recruited from Jiangxi Provincial People's Hospital (Nanchang, China) between September 2018 and August 2019. The distance between normal adjacent tissue and tumour tissue was 3 cm. The inclusion/exclusion criteria were as follows: i) Patients who underwent primary surgical resection and had histopathologically confirmed LUAD; ii) patients who were ≥18 years old, iii) patients who had no pulmonary metastases or other concomitant cancers; and iv) patients for whom informed consent was provided for the use of tissues. Detailed patient clinical information was collected retrospectively, including age (46 males and 29 females), sex (mean age, 61.6 years; range 40-83 years), tumour location, tumour size, histological differentiation grade, lymph node metastasis, and Tumour-Node-Metastasis (TNM) stage. All patients were staged according to the 8th edition of the TNM staging system for lung cancer (21). There was missing data in tumour
Immunohistochemistry (IHC). Immunohistochemical staining was performed as described previously (22). Briefly, the tissue was fixed with 4% formaldehyde at room temperature for 24 h and then embedded with paraffin and tissue specimens were cut into 5-µm serial sections. Then, the sections were dewaxed and dehydrated in a xylene and different concentration of alcohol solution (100, 95, 90, 80%). Heat mediated antigen retrieval was performed with Tris/EDTA buffer pH 9.0. The endogenous peroxidase activity was then blocked using 0.3% hydrogen peroxide for 10 min at room temperature. The sections were cooled and blocked by incubating with normal goat serum for 1 h at room temperature. The sections were then incubated overnight at 4°C with rabbit anti-human MELK antibody (cat. no. ab129373; 1:200; Abcam). The sections were next incubated with biotinylated secondary goat anti-rabbit polyclonal antibody (cat. no. ab6720; 1:800; Abcam) for 30 min at room temperature, followed by incubation with streptavidin horseradish peroxidase complex. Finally, sections were visualized by 3,3’-diaminobenzidine staining. The results of immunohistochemical staining were calculated according to the immunoreaction score (IRS) and evaluated by 2 pathologists in a double-blind manner as described previously (23). Briefly, the range of the IRS score was from 0-12, which was calculated as staining intensity (SI) x percentage of positive cells (PP). High expression of MELK was represented by IRS >4, while low expression of MELK was represented by IRS ≤4. For haematoxylin and eosin staining (H&E), which was also performed in a double-blind manner, 5-µm sections from the paraffin blocks were stained with H&E for 5 min at room temperature by one pathologist, and the other pathologist observed the results under an optical microscope (Olympus Corporation).

Statistical analysis. Oncomine, TCGA and GEPIA data were analysed using an unpaired Student's t-test for the location, tumour size, histological differentiation, T stage, N stage, M stage and TNM stage. The research was approved by the Ethical Committee of Jiangxi Provincial People's Hospital (Jiangxi, China) (approval no. 2018070). Informed consent was provided by the clinicians and obtained from the patients who provided written informed consent for the usage of the tissues for research.

Figure 2. Levels of MELK expression in LUAD based on data from the Oncomine database. (A) Meta-analysis of the 7 analyses on MELK mRNA levels in LUAD identified via Oncomine. (B) mRNA expression of MELK in LUAD using Oncomine database. (a) MELK mRNA expression in LUAD in Hou Lung dataset. (b) MELK mRNA expression in LUAD in Landi Lung dataset. (c) MELK mRNA expression in LUAD in Okayama Lung dataset. (d) MELK mRNA expression in LUAD in Beer Lung dataset. (e) MELK mRNA expression in LUAD in Selamat Lung dataset. (f) MELK mRNA expression in LUAD in Stearman Lung dataset. (g) MELK mRNA expression in LUAD in Su Lung dataset. The X axis of the plot represents the normal vs. cancer group, and the Y axis represents log2-transformed, median/mean centred mRNA expression. The line in the middle represents the median value. LUAD, lung adenocarcinoma; MELK, maternal embryonic leucine zipper kinase.
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Comparison of 2 groups or ANOVA followed by a post hoc Tukey's honest significance difference (HSD) test for multiple comparisons conducted to compare the differential mRNA and protein expression of MELK. The patients' clinical data are presented as the mean ± S.E.M. SPSS 19.0 software (IBM Corp.) was used to analyse the clinical data using the unpaired t-test and χ² tests as appropriate. Kaplan-Meier plotter, GEPIA and TCGA analyses were performed to assess the effect of MELK on the overall survival time of patients with LUAD. Log-rank P-values and HRs with 95% CIs were calculated and displayed on the webpage. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression levels of MELK in multiple cancer types. MELK expression in different cancer types was assessed using the Oncomine database (Fig. 1). In the cancer vs. cancer comparison, there were 695 cancer histology comparisons and 268 multi-cancer comparisons, and both had the same number of significant unique samples regardless of if they were in the MELK high or low expression groups. In addition, in the cancer vs. normal adjacent comparison, there were 427 unique comparisons for MELK expression in various tumours and 97 comparisons were unique. Among them, 91 analyses demonstrated higher expression of MELK in 18 kinds of malignant...
tumours compared with adjacent normal tissues, including bladder, brain and central nervous system (CNS), breast, cervical, colorectal, oesophageal, gastric, head and neck, kidney, liver, lung, ovarian and pancreatic cancer types, as well as melanoma, lymphoma, leukaemia, sarcoma and other cancer types. The next 6 analyses, including analyses in brain and CNS cancer, breast cancer, leukaemia and other cancer types, showed lower expression of MELK (Fig. 1). In addition, there were numerous more significant comparisons in 4 types of tumours, namely, breast cancer (15 unique analyses), colorectal cancer (12 unique analyses), sarcoma (11 unique analyses) and lung cancer (9 unique analyses), compared with other types of tumours, wherein MELK expression in tumours was higher compared with that in normal tissues. Lung cancer is the leading cause of cancer incidence and mortality worldwide, and LUAD is the most prevalent subtype (2); therefore, the role of MELK in LUAD was further evaluated.

Expression of MELK in LUAD in different databases. A meta-analysis of 7 studies of MELK mRNA levels in LUAD was performed using the Oncomine database. As demonstrated, there was a higher level of MELK in LUAD tissues compared with that in normal tissues in different datasets (Fig. 2A and B). To verify the aforementioned results, TCGA and GEPIA datasets were used to analyse MELK expression in patients with LUAD. As expected, higher levels of MELK were observed in LUAD compared with those in adjacent normal tissues both in TCGA and GEPIA datasets (Fig. 3A and E). In addition, the mRNA expression of MELK was assessed with regard to the different clinical parameters of LUAD, including sex, age, lymph node metastasis and tumour stage. MELK expression levels were significantly higher both in male and female patients with LUAD compared with those in normal tissues, and higher levels of MELK mRNA expression were found in male compared with female patients with LUAD (Fig. 3B). Notably, the upregulation of MELK in LUAD only occurred in patients >40 years old (Fig. 3C). There was no significant difference in the expression of MELK between patients <40 years old and normal tissue (Fig. 3C). In addition, the expression of MELK in LUAD tissues had no correlation with lymph node metastasis, but all were higher than normal group (Fig. 3D). The mRNA expression of MELK in different stages of LUAD was analysed and the results demonstrated that the levels of MELK increased gradually with tumour grade, representing a possible role of MELK in cancer progression and invasion (Fig. 3F). Overall, the aforementioned findings were consistent, suggesting that the expression of MELK in tumours was higher compared with that in normal lung tissues.

MELK protein expression is upregulated in tumour tissue compared with normal tissue from patients with LUAD. Tumour and matched adjacent normal tissues were collected

Figure 4. Changes in the expression of MELK protein in tumour and normal tissues from patients with LUAD. (A) Haematoxylin and eosin staining of LUAD tissues (original magnifications, ×50 and ×200). (B) MELK expression in LUAD was detected by immunohistochemistry (original magnifications, ×50 and ×200). (C) Quantification of positive staining for MELK in LUAD and normal tissues. (D) High expression of MELK in LUAD (n=75). A Student’s t-test was used to compare the expression of MELK between LUAD and normal tissues. *P<0.05 vs. non-cancer. LUAD, lung adenocarcinoma; MELK, maternal embryonic leucine zipper kinase.
from 75 patients with LUAD to evaluate the protein expression of MELK. Morphological changes were observed between tumour and matched normal tissues from patients with LUAD using H&E staining. Matched normal tissues showed normal histological status and consolidation and less alveoli were presented in LUAD tissues (Fig. 4A). Subsequently, the levels of MELK protein in these tissues were assessed using IHC. The analysis suggested that MELK protein was increased in patients with LUAD (Fig. 4B and C), and the high expression rate of MELK was 76% (57/75; Fig. 4D). The association between MELK protein expression and clinicopathological features in 75 patients with LUAD was compared, and the results demonstrated that a high level of MELK was associated with M stage and TNM stage (Table I). However, no significant difference was found between MELK protein expression and age, sex, histological differentiation, T stage, N stage, M stage, and TNM stage. MELK, maternal embryonic leucine zipper kinase; TNM, Tumour-Node-Metastasis; T, tumour; N, node; M, metastasis.

**Table I. Association between MELK protein expression and clinicopathological features in patients with lung adenocarcinoma (n=75).**

| Clinicopathological features | Patients, n (%) | MELK levels, n (%) | P-value |
|-----------------------------|-----------------|--------------------|---------|
|                             | Low             | High               |         |
| Age, years                  |                 |                    |         |
| <60                         | 7 (38.9)        | 25 (43.9)          | >0.999  |
| ≥60                         | 11 (61.1)       | 32 (56.1)          |         |
| Sex                         |                 |                    | 0.257   |
| Male                        | 9 (50.0)        | 37 (64.9)          |         |
| Female                      | 9 (50.0)        | 20 (35.1)          |         |
| Tumour location             |                 |                    | 0.480   |
| Left                        | 7 (41.2)        | 21 (36.8)          |         |
| Right                       | 10 (58.8)       | 36 (63.2)          |         |
| Tumour size, cm             |                 |                    | 0.611   |
| <5                          | 9 (60.0)        | 22 (59.5)          |         |
| ≥5                          | 6 (40.0)        | 15 (40.5)          |         |
| Histological differentiation |                 |                    | 0.088   |
| No                          | 1 (7.7)         | 1 (3.7)            |         |
| Low                         | 2 (15.4)        | 14 (51.9)          |         |
| Moderate                    | 5 (38.5)        | 9 (33.3)           |         |
| High                        | 5 (38.5)        | 3 (11.1)           |         |
| T stage                     |                 |                    | 0.057   |
| T1-2                        | 12 (75.0)       | 18 (47.4)          |         |
| T3-4                        | 4 (25.0)        | 20 (52.6)          |         |
| N stage                     |                 |                    | 0.382   |
| N0                          | 9 (56.3)        | 17 (47.2)          |         |
| N1-3                        | 7 (43.8)        | 19 (52.8%)         |         |
| M stage                     |                 |                    | 0.032   |
| M0                          | 13 (72.2)       | 24 (43.)           |         |
| M1                          | 5 (27.8)        | 31 (56.4)          |         |
| TNM stage                   |                 |                    | 0.045   |
| I-II                        | 11 (84.6)       | 14 (51.9)          |         |
| III-IV                      | 2 (15.4)        | 13 (48.1)          |         |

*P<0.05. The χ² test was used to analyse the data. There was missing data in tumour location, tumour size, histological differentiation, T stage, N stage, M stage, and TNM stage. MELK, maternal embryonic leucine zipper kinase; TNM, Tumour-Node-Metastasis; T, tumour; N, node; M, metastasis.*

**High MELK mRNA expression in patients with LUAD is associated with a poor prognosis.** Kaplan-Meier plotter, GEPIA and TCGA were used to analyse the prognosis of patients with LUAD with high and low MELK expression levels. The association between MELK mRNA level and OS in LUAD patients was analysed using the Kaplan-Meier plotter. The analysis demonstrated that high MELK expression was negatively associated with OS time (Fig. 5A). Similar trends were observed in the GEPIA and TCGA datasets, and upregulation of MELK increased the risk of mortality (Fig. 5B-D).
Overall, these results suggested that MELK upregulation decreases survival in patients with LUAD.

**Discussion**

MELK is a highly conserved serine/threonine kinase that was originally cloned from mice and is expressed in a wide range of early embryonic cellular stages (9). In recent years, MELK has been identified as a modulator of intracellular signalling and it mediates various cellular and biological processes, including the cell cycle, cell proliferation, apoptosis, cell renewal, gene expression and oncogenesis (15). In the present study, it was found that the expression of MELK was higher in tumour tissues compared with that in normal lung tissues, and that increased MELK expression was associated with a poor prognosis in patients with LUAD. The findings of the present study are consistent with those of previous studies, indicating that MELK may be a promising therapeutic target in LUAD (1,13,24).

The expression of MELK is negatively associated with the survival rate of patients with cancer (7‑9,11,25). In addition, the selective MELK inhibitor OTS167 has been shown to inhibit proliferation and promote apoptosis in a variety of tumours, leading to improved prognosis (8,9). Hence, MELK has been identified as a novel marker for predicting cancer outcomes and as a potential therapeutic target for some cancer types. Notably, MELK is 1 of 16 hub genes from the GEO database that were significantly associated with a worse
5-year OS rate of patients with lung cancer analysed using GEPIA (1). In addition, Zang et al (15) found that MELK mRNA expression level in tumour tissues was significantly higher compared with that in normal/benign tissue analysed using data from the TCGA and GEO databases, and that it was negatively associated with prognosis in patients with LUAD when subjected to Kaplan-Meier analysis. Inoue et al (24) confirmed that inhibition of the expression of MELK increased apoptosis and decreased the proliferation of SCLC cells. To further identify the role of MELK in LUAD and approve its use as a biomarker, additional data are needed for validation, including data from different databases and clinical samples. In the present study, the mRNA and protein expression levels of MELK in LUAD were analysed using Oncomine, TCGA and GEPIA, and immunohistochemistry, respectively. In the present study, results from the bioinformatics and immunohistochemistry analyses suggested that the MELK expression was higher in LUAD tissues compared with that in normal lung tissues. In the present study, no association was found between MELK expression and sex, age, tumour location, tumour size, histological differentiation, T stage or N stage in clinical samples. However, in TCGA samples, males had higher expression of MELK compared with females and there was no difference observed between normal tissue and tumour tissue from patients with LUAD 21-40 years old. In GEPIA samples, the levels of MELK increased gradually with tumour grade. These differences between clinical samples and databases may be due to the small sample size. In addition, in the present study, Kaplan-Meier, GEPIA and TCGA survival analyses were all used to evaluate prognosis and the result suggested that high expression of MELK was associated with poor OS probability in patients with LUAD. The findings of the present study and those of previous studies are consistent, and all demonstrate that MELK may serve as a potential diagnostic biomarker or therapeutic target for lung cancer (1,13,15,24).

MELK has been reported to participate in tumour progression via the JNK, p53, Bcl-G and forkhead box protein M1 (FOXM1) signalling pathways, which are all extremely important in multiple human cancer types (8,25-28). Gu et al (28) indicated that MELK regulates glioma cell growth via the JNK/p53 pathway. MELK triggers carcinogenesis through inhibition of the pro-apoptotic protein Bcl-G in breast cancer and hepatocarcinoma (25,27). Inhibition of MELK induces G2/M arrest and reduces cell proliferation by decreasing FoxM1 phosphorylation and upregulating p53 expression in chronic lymphocytic leukaemia (8). In addition, MELK is also reported to have critical roles in the formation or maintenance of cancer stem cells, which are associated with cancer recurrence (11,12). Researchers have also demonstrated that high levels of MELK contribute to cancer progression and stem cell maintenance in SCLC, and that inhibition of MELK increases apoptosis and suppresses the growth of cancer cells by reducing the activity of FoxM1 (24). However, the molecular mechanism of MELK in the progression of LUAD remains unclear. Whether the action of MELK in LUAD is the same as that in SCLC and other cancer types needs further investigation.

The present study has several limitations. Firstly, only 75 LUAD samples were used and a larger sample size is needed for future research. Secondly, only bioinformatics analysis was used to calculate the survival rate of patients with LUAD with high and low expression of MELK and further studies are required to verify the findings of the present study and increase accuracy. Thirdly, the present study mainly discussed MELK expression and prognosis in patients with LUAD, and the detailed molecular mechanism of MELK involvement in LUAD was not explored. All these limitations need to be investigated in future studies.

In summary, the present study demonstrated that MELK is highly expressed in LUAD and that there is a negative association between MELK expression and prognosis in affected patients. MELK may serve as a potential diagnostic marker and therapeutic target in LUAD; however, more studies are needed to investigate the potential mechanisms of MELK in this disease.

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Availability of data and materials
The three datasets generated and/or analyzed during the current study are available in the Oncomine databases (https://www.oncomine.org), The Cancer Genome Atlas database (https://tcga-data.nci.nih.gov/tcga/), the GEPIA database (http://gepia.cancer-pku.cn) and Kaplan-Meier Plotter database (http://kmplot.com/analysis).

Authors' contributions
SC and ZX designed the study. ZL, XC, XW, HT and LY collected the data. SC, LY, and HT analysed the data. SC, LY and ZX drafted and revised the manuscript for important intellectual content. All authors have read and approved the manuscript.

Ethics approval and consent to participate
The research was approved by the Ethical Committee of Jiangxi Provincial People's Hospital (Nanchang, China) (approval no. 2018070), informed consent was provided by the clinicians and obtained from the patients who provided written informed consent for the usage of the tissues for research.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.
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