Abstract. Osteosarcoma is the most common primary malignancy of bones and frequently affects young children and adolescents. There are several challenges associated with treating osteosarcoma owing to the aggressiveness of the disease, as well as the risk of chemoresistance. Numerous studies are being performed with the aim of identifying improved prognostic and therapeutic markers for this malignancy. Maternal embryonic leucine zipper kinase (MELK) is an oncogene that has been studied in several types of cancer in recent years. In the present study, the expression of MELK in osteosarcoma and normal tissue samples was examined, and the effects of MELK expression on osteosarcoma cellular proliferation, metastasis, the cell cycle and apoptosis were demonstrated using CCK-8, wound healing, migration and invasion and apoptosis assays. The role of MELK in cancer progression in osteosarcoma was determined, revealing the association between MELK expression and prognosis of osteosarcoma. It was demonstrated that knockdown of MELK resulted in reduced proliferation, migration and invasion in vitro along with potentiation of apoptosis and cell cycle arrest. Furthermore, the effect of the targeted MELK inhibitor, OTSSP167, on tumor progression of osteosarcoma in vitro and in vivo was assessed. Mechanistically, it was demonstrated that MELK promoted osteosarcoma proliferation and metastasis by regulating PCNA and MMP9 expression via the PI3K/Akt/mTOR signaling pathway. Thus, the present study revealed the oncogenic role played by MELK, and established MELK as a valuable prognostic and therapeutic marker in osteosarcoma.

Introduction

Osteosarcoma is the most common primary malignancy of bones, and accounts for ~2% of all childhood malignancies (1-3). It is primarily diagnosed in teens and adolescents and in the elderly population over 65 years (4,5). Osteosarcoma is a markedly aggressive cancer with a high capacity to form distant metastases, and there is a high rate of resistance to the traditional therapeutic approaches (3). There have been only minimal advances made in recent decades in the treatment of osteosarcoma (5). Although multimodal therapy has revealed improved outcomes in patients with osteosarcoma, this is not the case for the patients who present with metastases at the initial diagnosis (6). Therefore, it is necessary to study the pathogenesis and metastatic mechanisms underlying the development of osteosarcoma, to identify novel therapeutic targets for improving the treatment and prognosis.

Maternal embryonic leucine zipper kinase (MELK) belongs to the Snf1/AMPK kinase family and is also referred to as murine protein K38 (MPK38) or Eg3 protein (7,8). It is highly expressed in the egg and in the pre-implanted embryo in mice, and influences embryonic development (9). MELK is a highly conserved AMP-activated Ser/Thr protein kinase (7), and its expression is upregulated in several types of cancer, including glioblastoma, melanoma, breast cancer, ovarian cancer, gastric cancer and colorectal cancer (10-15). MELK performs various functions in different types of cancer, which promote the development and progression of cancer (13,16,17). However, the role and mechanism of MELK in osteosarcoma has not been studied, to the best of our knowledge. In the present study, the effects of MELK on crucial functions, such as proliferation, metastasis and apoptosis, which promote the progression of osteosarcoma, was studied. OTSSP167, a MELK inhibitor, is already in phase I clinical trials for the treatment of solid tumors that do not respond to any other treatment (18,19), and if successful may serve as therapy for the treatment of several different types of cancer. Therefore, the role of the novel molecular inhibitor of MELK, OTSSP167, in osteosarcoma was also explored.

The aim of the present study was to determine the roles and the underlying mechanisms of MELK in osteosarcoma. MELK expression was determined to be upregulated in osteosarcoma compared with the normal control samples, and the increased expression was associated with a less
favorable prognosis. Through analysis of the clinicopathological characteristics, it was revealed that there was a positive association between MELK expression with metastasis and a poor response to chemotherapy. A series of in vitro and in vivo experiments were performed to establish the role of MELK in crucial functions such as proliferation, metastasis, cell cycle progression and apoptosis of osteosarcoma. Furthermore, the role and therapeutic effects of OTSSP167 in osteosarcoma were also studied in vitro and in vivo. The results revealed that MELK promoted osteosarcoma proliferation and metastasis by regulating PCNA and MMP9 expression through the PI3K/Akt/mTOR signaling pathway. Collectively, these results indicated that MELK may be a novel prognostic marker and therapeutic target for treatment of osteosarcoma.

Materials and methods

Analysis of public datasets. Gene expression data including 84 cases of osteosarcoma were acquired from the Therapeutically Applicable Research to Generate Effective Treatments (TARGET) program, which is accessible publicly (https://portal.gdc.cancer.gov/projects). The relative differences in MELK expression were analyzed using X-tile software (version 3.6.1; Yale University School of Medicine) to determine the thresholds for producing the most significant log-rank test P-values, which segregated the data into MELK-low and MELK-high groups. The differences in survival between the low- and high-expression groups were evaluated using Kaplan-Meier analysis.

Tissue samples and cell lines. A total of 25 osteosarcoma tissue samples that had not received any neoadjuvant chemotherapy and 5 osteoblastoma tissue samples were collected from patients who were treated at Qilu Hospital of Shandong University from January 2010 to June 2014. All patients signed informed consent to participate in the present study, and the study was approved by the Ethics Committee of Qilu Hospital of Shandong University. The small number of samples is due the fact that most osteosarcoma patients receive preoperative neoadjuvant chemotherapy. MNNG/HOS and hFOB1.19 cell lines were purchased from the American Type Culture Collection. MNNG/HOS cells were maintained in DMEM with 5% FBS (Gibco; Thermo Fisher Scientific, Inc.) and hFOB1.19 cells were maintained in a 1:1 mixture of Ham's F12 medium and DMEM supplemented with 10% FBS. All the cells were incubated at 37°C with 5% CO2, MELK inhibitor (OTSSP167; cat. no. S7159) and PI3K inhibitor (LY294002; cat. no. S1105) were purchased from Selleck Chemicals.

Immunohistochemistry. Immunohistochemical staining was performed on tissue sections (4-µm thick) of formalin-fixed at room temperature for 48 h, paraffin-embedded osteosarcoma tissue samples. Xylene and ethanol were used to deparaffinize and rehydrate the tissue slides. Antigen retrieval was performed using sodium citrate buffer solution (pH 6.0) maintained at a sub-boiling temperature for 15 min. Endogenous peroxidase and nonspecific binding were blocked using 3% hydrogen peroxide for 15 min and goat serum (SP-kit: cat. no. SP9000; Beijing Zhongshan Golden Bridge Biotechnology) for 30 min, respectively at room temperature. Tissues were incubated with anti-MELK primary antibody (1:250; cat. no. bs-12201R; Bioss) in a humid chamber overnight at 4°C. Expression was detected using a I-View 3,3′-diaminobenzidine staining technique. To evaluate the staining intensity, semiquantitative evaluation of protein levels in tissues was performed using the H-score technique (range, 0-3) (20). The staining intensity was graded as: 0; none; 1; weak; 2; moderate; and 3, strong. The H-score was calculated using the formula: H-score=ΣPi x I; where I represents the staining intensity and Pi the percentage of cells at each level of intensity. An H-score <1.5 was classified as low, and a H-score ≥1.5 was classified as a high protein expression level.

Transient transfection. Small interfering (si)RNAs and the corresponding negative control (NC) were used to transiently knockdown expression of MELK. The siRNAs were purchased from (Shanghai GenePharma, Co., Ltd.). RNAi-mediated knockdown was performed using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol. The sequences of the siRNA used were: si-MELK, 5’-GAC AUC CUA UCU AGC UGC A-3’ and si-NC, 5’-UUCUCCGAACGUGUCAGUTT-3’.

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from osteosarcoma cells using TRIzol® reagent (Ambion; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol. The cDNA was synthesized using a One Step PrimeScript miRNA cDNA Synthesis kit (Takara Bio, Inc.). qPCR was performed using a SYBR Green Premix Ex Taq II (Takara Bio, Inc.) with a StepOne Plus Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The reaction conditions were as follows: Denaturation at 95°C for 5 sec, followed by 40 cycles of annealing at 60°C for 10 sec and an extension at 72°C for 30 sec. GAPDH expression was used as the endogenous control for the detection of miRNA expression levels. The 2△ΔCt was used to calculate the relative gene expression level (21). The sequences of the primers used were: GAPDH forward, 5’-CAGAGGGCATG TTGATGAT-3’ and reverse, 5’-GAAGGCTGGGCCTCA TTGT-3’; and MELK, forward 5’-TCTCCAGTAGCTTCTG CTTT-3’ and reverse 5’-TGATCCAGGGATGTTCAATAGA3’. Primers were purchased from Sangon Biotech, Co., Ltd.

Western blotting. Cells and tissues were harvested and lysed using RIPA Lysis Buffer (Beyotime Institute of Biotechnology) with PMSF (1%). Protein samples were incubated for 30 min on ice and sonicated, after which the cell debris were removed by centrifugation at 12,000 x g. The protein concentration was determined using a bicinchoninic acid assay kit (Thermo Fisher Scientific, Inc.). Protein samples (30 µg) were resolved by SDS-PAGE (5% stacking gel and 10% separating gel) and transferred to a PVDF membrane (EMD Millipore). The membrane was blocked with 5% non-fat milk at room temperature for 1 h, incubated overnight at 4°C with the primary antibody, and subsequently incubated with horse-radish peroxidase-conjugated anti-rabbit (dilution 1:5,000; cat. no. TA130023; OriGene Technologies, Inc.) secondary antibody for 2 h. Signals were visualized using enhanced chemiluminescence reagent and ImageQuant LAS 4000 (GE
Healthcare Life Sciences). ImageJ software version 1.47v (National Institutes of Health) was used for densitometric measurement.

The antibodies used were: Phosphorylated (p)-PI3K p85 (1:1,000; product no. 4228; Cell Signaling Technology, Inc.), total PI3K p85 (1:1,000; product code ab40755; Abcam), p-Akt(S473) (1:1,000; product no. 9271S; CST Biological Reagents Co., Ltd.), GAPDH (1:5,000; cat. no. AF7021; Affinity Biosciences), MELK (1:1,000; product code ab108529; Abcam), mTOR (1:1,000; product no. 2972S; CST Biological Reagents, Co., Ltd.), p-mTOR (1:1,000; product no. 5536S; CST Biological Reagents, Co., Ltd.), PCNA (1:750; cat. no. AF0239) and MMP9 (1:750; cat. no. AF5228; both from Affinity Biosciences).

Cell proliferation assay. The proliferative capacity of cells was assessed using a Cell Counting Kit-8 (CCK-8) assay (BestBio). Each cell line was seeded in quintuplicate into 96-well plates (1-2x10^5 cells/well) for 1-5 days. At the specified time-points, 10 µl CCK-8 reagent was added to each well, and the cells were incubated for an additional 4 h at 37°C. Subsequently, the absorbance values at 450 nm were measured using a Varioskan Flash microplate reader (Thermo Fisher Scientific, Inc.). Cell proliferation was measured in si-MELK and si-NC transfected cells, and in cells treated with 40 nM OTSSP167. For the latter, cells were initially treated for 48 h with OTSSP167.

Cell viability assay. A total of 5x10^3 cells/well were seeded in quintuplicate into 96-well plates. The plate was incubated at 37°C for 24 h, and subsequently treated with various concentrations of OTSSP167 (0, 2, 4, 8, 16, 32 and 64 nM) for 48 h. For evaluation of cell viability, 10 µl CCK-8 reagent was added to each well and incubated at 37°C for 4 h. The cell viability was determined by measuring the absorbance at 450 nm using Varioskan microplate reader.

Migration and invasion assay. Cellular invasion and migration were analyzed using Boyden chamber-style cell culture inserts with and without Matrigel, respectively (BD Falcon; BD Biosciences). Osteosarcoma cells (1-2x10^5 cells) were seeded in the upper chambers of the Transwell inserts (24-wells, 8 µm pores; BD Falcon) with 200 µl serum-free media. The lower chambers were filled with 500 µl culture media supplemented with 10% FBS as a chemoattractant. After 12-48 h, cells on the lower surface of the membrane were fixed at room temperature in methanol for 20 min, stained at room temperature with 0.1% crystal violet for 30 min, and counted using a light microscope (magnification, x200).

Wound healing assay. Cell migration was measured using a wound healing assay. MNNG/HOS cells were used for the assay at a density of 5x10^5/well. The scratch was generated using 200-µl pipette tip. The culture medium was replaced with serum-free DMEM after the generation of a scratch wound, and wound closure was imaged 48 h later under a light microscope (magnification, x100). Wound closure was analyzed and is represented as percent closure. The experiments were performed in triplicate. Wound healing assays were performed on si-MELK and si-NC transfected cells, and cells treated with 10 nM OTSSP167.

Cell cycle distribution and apoptosis assay. Cell cycle synchronization was achieved by serum starvation and thymidine double blocking (22). Subsequently, the cells were harvested and stained with 5 µl propidium iodide at 4°C for 30 min in the dark according to the manufacturer's protocol (BestBio). Flow cytometry was performed using a FACScan flow cytometer (BD Biosciences).

Apoptosis was detected using an Annexin V-APC and 7-AAD kit (BioGems International, Inc.) according to the manufacturer's protocol, and flow cytometry was performed on the cells. MNNG/HOS cells were harvested 48 h after transfection or treated with OTSSP167 (10 nM) and subsequently harvested using EDTA-free trypsin, centrifuged at 800 x g for 5 min; and, after washing twice with cold PBS, the cells were resuspended at a concentration of 1x10^6/ml and stained with 5 µl Annexin V-APC and 5 µl 7-AAD at room temperature for 15 min in the dark. The cells were analyzed by flow cytometry (FACSCalibur) and the results were analyzed using FlowJo software (version 10; TreeStar, Inc.).

Animal experiments. Eight pathogen-free female Balb/c nude mice (weighing 10-14 g; 4 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd and housed in standard sterilized conditions in individually ventilated cages (IVCs) with high efficiency particulate air (HEPA) filters at an ambient temperature of 30-31°C, humidity of 50-60% with 12-h light/dark cycle and adequate access to food and water. The health and behavior of the animals were monitored every 3 days. All animal experiments were approved by the Ethics Committee of Qilu Hospital, Shandong University.

For tumorigenesis assays, 1x10^7 MNNG/HOS cells, resuspended in 200 µl PBS, were injected subcutaneously into the armpits of the mice. The tumor volume was measured daily using a Vernier caliper, with the following formula: ½ x a x b^2 where ‘a’ and ‘b’ represent the length and width of the tumor, respectively. After 7 days, when the tumor volumes reached ~100 mm^3, the tumor-bearing mice were orally fed Vehicle or OTSSP167 (10 mg/kg, daily). The tumor volumes were measured daily using Vernier calipers. After 7 days, when the maximum tumor volumes reached ~1,000 mm^3 the mice were euthanized using cervical dislocation method and then the growth of the tumors was examined. Following excision, the tumors were preserved at -80°C for western blot analysis.

Statistical analysis. Data were analyzed using independent samples t-test, log-rank test, or Fisher's exact test. Fisher's exact test was used to analyze the relationship between MELK expression and clinicopathological data of osteosarcoma patients. Overall survival (OS) curves were plotted using the Kaplan-Meier method and analyzed using a log-rank test. Quantitative data were analyzed using a Student's t-test. Data are expressed as the mean ± the standard deviation of three independent measurements. Statistical analysis was performed using SPSS version 18.0 (SPSS Inc.) and GraphPad Prism version 5.0 (GraphPad Software Inc.). Two-tailed P<0.05 was considered to indicate a statistically significant difference.
Results

*MELK expression is upregulated in osteosarcoma and its overexpression is associated with a less favorable prognosis.*

The expression levels of MELK were assessed in 25 osteosarcoma tissue samples and 5 osteoblastoma samples using immunohistochemistry (Fig. 1A). H-score evaluation revealed significantly higher protein expression levels in osteosarcoma tissues compared with the osteoblastoma tissues (Fig. 1B).

Of the 25 samples, 40% exhibited high staining and 60% exhibited low staining, whereas in the osteoblastoma samples, none of the tissues showed MELK expression (Fig. 1A). MELK mRNA expression levels were also compared between MNNG/HOS and hFOB1.19 cells. MELK expression was significantly higher in the MNNG/HOS cells compared with the hFOB1.9 cells (Fig. 1C). Subsequently, the association between MELK expression and various clinicopathological characteristics of the patients were assessed. As revealed in
Table I. Association between MELK expression and clinicopathological data of osteosarcoma patients.

| Parameters                  | N    | High n=10 (40%) | Low n=15 (60%) | P-value |
|-----------------------------|------|-----------------|----------------|---------|
| Age (years)                 |      |                 |                |         |
| <18                         | 14   | 5               | 9              | 0.6968  |
| ≥18                         | 11   | 5               | 6              |         |
| Sex                         |      |                 |                | 0.2262  |
| Male                        | 13   | 7               | 6              |         |
| Female                      | 12   | 3               | 9              |         |
| Location                    |      |                 |                | 0.1500  |
| Extremity                   | 23   | 8               | 15             |         |
| Trunk                       | 2    | 2               | 0              |         |
| Histological subtype       |      |                 |                | 0.8889  |
| Osteoblastic                | 8    | 2               | 6              |         |
| Fibroblastic                | 4    | 3               | 1              |         |
| Chondroblastic              | 7    | 4               | 3              |         |
| Others¹                     | 6    | 1               | 5              |         |
| Metastasis                  |      |                 |                | 0.0414b |
| Yes                         | 13   | 8               | 5              |         |
| No                          | 12   | 2               | 10             |         |
| Surgery                     |      |                 |                | 0.4139  |
| Amputation                  | 14   | 7               | 7              |         |
| Limb salvage                | 11   | 3               | 8              |         |
| Response to chemotherapy    |      |                 |                | 0.0154b |
| Good                        | 13   | 2               | 11             |         |
| Poor                        | 12   | 8               | 4              |         |
| Enneking stage              |      |                 |                | 0.472   |
| I                           | 2    | 0               | 2              |         |
| II                          | 20   | 8               | 12             |         |
| III                         | 3    | 2               | 1              |         |

P-values were calculated by Fisher's exact test. ¹Others: Telangiectatic, small-cell, parosteal or undifferentiated osteosarcoma. bP<0.05. MELK, maternal embryonic leucine zipper kinase.

Table I higher expression levels of MELK were associated with metastasis and the response to chemotherapy. Kaplan-Meier survival curves revealed that OS was shorter in patients with high MELK expression levels, based on the data obtained from TARGET (Fig. 1E), in agreement with the present analysis. Thus, high expression levels of MELK were associated with less favorable OS in patients (Fig. 1D).

**MELK knockdown reduces proliferation in vitro.** Si-MELK was used to knock down the expression of MELK in MNNG/HOS cells. The mRNA and protein expression levels of MELK following knockdown are presented in Fig. 2A and B. To determine the effect of MELK on the proliferation of osteosarcoma cells, a proliferation assay was performed using MNNG/HOS cells transiently transfected with si-NC or si-MELK. As revealed in Fig. 2C, knockdown of MELK expression significantly decreased proliferation. OTSSP167, a MELK inhibitor, was used to assess its effects on proliferation. First, the IC_{50} of OTSSP167 was determined, which was determined to be 40 nM/ml (Fig. 2D). There was also a dose-dependent decrease in MELK expression levels following treatment with OTSSP167 (Fig. 2E). Using 40 nM OTSSP167, the proliferation of cells treated with vehicle or OTSSP167 was assessed. As revealed in Fig. 2F, there was a significant decrease in the proliferation of cells treated with OTSSP167. These in vitro experiments demonstrated that MELK serves a substantial role in the proliferation of osteosarcoma cells.

**OTSSP167 inhibits tumorigenesis in vivo.** To determine the role of MELK in tumorigenesis, MNNG/HOS cells were subcutaneously injected into the armpits of nude mice. Tumor growth was closely monitored and tumor volume was measured daily using a Vernier caliper, using the aforementioned formula. When the tumor volume reached
~100 mm³, the mice were randomly divided into two groups; one group was orally fed OTSSP167 (10 mg/kg) daily whilst the other group was fed vehicle. After 7 days, the mice were euthanized, and the tumors were excised and analyzed. The tumor volume exhibited a gradual decrease in the group fed OTSSP167 compared with the control group fed vehicle. There was a significant difference in the tumor weight between the two groups (Fig. 3A-D). To evaluate the results, the protein expression levels were assessed between the groups, and the expression of MELK was revealed to be lower in the mice fed OTSSP167 (Fig. 3E).

**MElk reduces apoptosis and promotes cell cycle progression in osteosarcoma cells.** The proportion of apoptotic MNNG/HOS cells following MELK knockdown was assessed and compared with the control cells. The results revealed that there was an increase in the total proportion of apoptotic cells and the portion of cells in early stage apoptosis when MELK expression was knocked down (Fig. 4A). Additionally, MELK knockdown resulted in inhibition of cell cycle progression in osteosarcoma cells (Fig. 4B). There was an increased proportion of cells in the G1 phase and a lower proportion of cells in the G2 and S phases in the MELK-knockdown cells compared
with the control. It was hypothesized that MELK exerted its function on osteosarcoma proliferation by inhibiting apoptosis and potentiating the cell cycle. Such effects were also demonstrated when MELK was inhibited by OTSSP167. Notably, the magnitude of increase in apoptosis and cell cycle inhibition were more pronounced with OTSSP167 (Fig. 4C and D).

**MELK knockdown decreases migration and invasion in vitro.** The role of MELK on the migration and invasion of osteosarcoma cells was assessed using Transwell assays with or without Matrigel, and wound healing assays. As revealed in Fig. 5A and B, MNNG/HOS cells exhibited reduced migratory and invasive capacities following MELK knockdown. When MELK was inhibited using the inhibitor OTSSP167, similar inhibitory effects were observed on the migration and invasion abilities (Fig. 5C and D). Thus, it was hypothesized that MELK serves a role in potentiating the migratory and invasive capacities of osteosarcoma cells. The clinicopathological analysis from immunohistochemistry also revealed a significant association between upregulated MELK expression and metastasis of osteosarcoma, in agreement with the results of the in vitro experiments.

**MELK promotes proliferation and metastasis of osteosarcoma through the PI3K/AKT/mTOR pathway.** The PI3K/AKT and the mTOR signaling pathways are considered principal pathways involved in several oncogenic mechanisms. In the present study, it was revealed that knocking down MELK expression resulted in reduced phosphorylation of PI3K, AKT and mTOR, and reduced protein expression levels of MMP9 and PCNA (Fig. 6A). Similar results were also observed with the use of OTSSP167 (Fig. 6B). Furthermore, in MNNG/HOS cell treated with LY294002 (20 µM), the expression of p-AKT, p-mTOR, PCNA and MMP9 were decreased (Fig. 6C). Collectively, these results indicated that MELK promoted proliferation and metastasis of osteosarcoma cells by modulating PCNA and MMP9 expression via the PI3K/AKT/mTOR pathway.

**Discussion**

There is an increasing body of literature showing that MELK expression is upregulated in several types of cancer (10-15), and its upregulated expression is associated with a less favorable prognosis in cancer (13,16,17). Similarly, MELK has been
demonstrated to exert its oncogenic functions by affecting cellular proliferation, cell cycle, apoptosis and metastasis (23,24). The present study is the first study, to the best of our knowledge, to reveal the role of MELK in the progression and metastasis of osteosarcoma.

In the present study, MELK expression was revealed to be significantly upregulated in osteosarcoma tissues compared with normal tissues. Compared with the hFOB1.19 cell line, MELK expression levels were also upregulated in the MNNG/HOS osteosarcoma cell line. Analysis of the clinicopathological characteristics revealed that upregulated expression of MELK was closely associated with metastasis and chemotherapy response in patients with osteosarcoma. Furthermore, MELK expression was associated with the OS of the patients; patients with high MELK expression levels had a worse prognosis than patients with low expression.

MELK expression was knocked down in the MNNG/HOS osteosarcoma cell line using si-MELK or the MELK inhibitor, OTSSP167. Knockdown or inhibition of MELK activity resulted in a significant decrease in cellular proliferation and metastatic behavior. MELK has previously been revealed to regulate proliferation, migration and invasion (11-17). In agreement with the previous studies, suppressing MELK in the MNNG/HOS osteosarcoma cell line reduced proliferation, migration and invasion. There was also a significant decrease in cell cycle progression and potentiation of apoptosis due to knockdown of MELK.

In addition to identifying the role of MELK in osteosarcoma, the underlying mechanism was also identified. PCNA has been revealed to be an indicator of cell proliferation, and to serve a principal role in the regulation of cellular proliferation and cell cycle progression (25,26). Furthermore, PCNA
is a known oncogene and its upregulation has been revealed to be associated with a poorer prognosis in patients with osteosarcoma (27).

In the present study, the expression levels of PCNA were revealed to be decreased following knockdown or inhibition of MELK. Thus, it was hypothesized that MELK may promote the growth of osteosarcoma by regulating PCNA expression.

MMPs are proteolytic enzymes that serve a role in extracellular matrix remodeling, affecting cell growth, differentiation, migration, invasion and angiogenesis (28). As an important member of the MMP family, MMP-9 has been revealed to affect tumor metastasis in several tumors, including osteosarcoma (29-31). The results of the present study revealed that there was a significant decrease in migration and invasion based on the Transwell and wound healing assays. Western blot analysis also revealed that MMP9 protein expression levels were lower in the cells when MELK expression was knocked down, indicating reduced invasive capacity.

The PI3K/AKT/mTOR pathway is associated with a variety of cellular functions and is known to serve a distinct role in cancer progression (32). In the present study, silencing of MELK resulted in marked suppression of p-PI3K, p-AKT, p-mTOR, PCNA and MMP9 expression. It was further revealed that a PI3K inhibitor decreased the expression of p-AKT, p-mTOR, PCNA and MMP9. Collectively, it was hypothesized that MELK affected the proliferation and migration of osteosarcoma cells by regulating PCNA and MMP9 expression via the PI3K/Akt/mTOR signaling pathway. Studies have previously revealed a link between PCNA and the PI3K/Akt/mTOR signaling pathway (33,34), and this pathway is known to be involved in regulation of migration and invasion in various types of cancer, possibly through regulation of crucial MMPs (35-37).

OTSSP167 is a potent MELK inhibitor, which has been demonstrated to possess antitumor properties in several types of cancer (15,16). OTSSP167 is a protein kinase inhibitor that prevents MELK phosphorylation and abrogates its downstream substrates (38). OTSSP167 is being developed as an anticancer drug for patients with solid tumors, which have not responded to established modes of treatment, and is emerging as a promising therapeutic for treatment of several types of solid tumors (20). A phase I clinical trial studying the efficiency of OTSSP167 was started in August of 2013 (18).

In the present study, OTSSP167 served an inhibitory role in osteosarcoma progression through suppression of MELK. When xenografted tumors were produced in nude mice and were orally fed OTSSP167, there was a gradual decrease in the size of tumors over time, whereas the control group, which was fed the vehicle alone, possessed tumors which grew over time. This is in agreement with the in vitro proliferation assays and strongly supports the hypothesis that MELK is responsible for tumor progression and its knockdown or inhibition by RNAi or OTSSP167, respectively, could reduce tumor progression. In recent decades, interest in studying inhibitors of PI3K, AKT and mTOR has increased. Several of these inhibitors are in various phases of clinical trials (39). Since the MELK inhibitor OTSSP167 has been revealed to downregulate the phosphorylation of these molecules, it is hypothesized that it may be used as a substitute for these inhibitors. However, additional studies are required for verification.

Figure 5. MELK knockdown reduces migration and invasion in vitro. (A) Wound healing assays revealed the reduced migratory potential of cells following knockdown of MELK. (B) Transwell assays revealed reduced migratory and invasive potential of MNNG/HOS cells following knockdown of MELK. (C) Migration (wound healing assay) and (D) migration and invasion (Transwell assays) were reduced in osteosarcoma cells following treatment with OTSSP167. "P<0.01, "**P<0.001. MELK, maternal embryonic leucine zipper kinase.
Figure 6. MELK promotes osteosarcoma proliferation and metastasis through the PI3K/AKT/mTOR pathway. (A) Western blot analysis of changes in protein expression following knockdown of MELK. Cells transfected with si-MELK exhibited lower expression levels of p-PI3K (Tyr485), p-AKT (Ser473), p-mTOR, MMP9 and PCNA. (B) Western blotting revealed lower protein expression levels of p-PI3K, p-AKT, p-mTOR, MMP9 and PCNA in the cells treated with OTSSP167 compared with the vehicle control. (C) PI3K inhibitor LY294002 decreased the expression levels of p-AKT, p-mTOR, PCNA and MMP9. GAPDH was used as the internal control. *P<0.05, **P<0.01, ***P<0.001. MELK, maternal embryonic leucine zipper kinase; si, small interfering; p-, phospho-.
In summary, MELK expression was upregulated in osteosarcoma, and this was associated with a poor prognosis. MELK increased proliferation and metastasis of osteosarcoma cells by modulating PCNA and MMP9 expression via the PI3K/AKT/mTOR pathway. The present study is the first to examine the role of MELK in osteosarcoma, the first to determine the underlying mechanism of MELK-mediated effects, and the first to determine the effect of OTSSP167 in suppressing osteosarcoma progression through inhibition of MELK. These results suggest that MELK may be used as a prognostic biomarker, and highlights its potential as a therapeutic target for the treatment of osteosarcoma.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request. The results published here are in whole or in part are based on data generated by the Therapeutically Applicable Research to Generate Effective Treatments (https://ocg.cancer.gov/programs/target) initiative, phs000468. The data used for this analysis are available at https://portal.gdc.cancer.gov/projects.

Authors' contributions

SFAJ and XW provided substantial contributions to the conception and design of the work. SFAJ and XW were responsible for the experimental procedure and clinical studies. KL and XL conducted the literature search. QY and JL provided the data acquisition and carried out the statistical analysis. SD drafted the work, edited the manuscript and revised it critically for important intellectual content. All authors critically revised and approved the final manuscript and agree to be responsible for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All patients signed informed consent to participate in the present study, and the study was approved by the Ethics Committee of Qilu Hospital of Shandong University. All animal experiments were approved by the Ethics Committee of Qilu Hospital, Shandong University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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