Mitotic spindle organizing protein 2A (MZT2A) promotes NSCLC cell viability and invasion by upregulating galectin-3-binding protein (LGALS3BP), increases AKT phosphorylation via the MOZART2 domain and predicts poor NSCLC prognosis

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Research

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Abstract

Background: Mitotic spindle organizing protein 2A (MZT2A) is localized at the centrosome and regulates microtubule nucleation activity in cells. This study assessed the role of MZT2A in non-small cell lung cancer (NSCLC).

Methods: Differential MZT2A expression was bioinformatically assessed using the Cancer Genome Atlas database, Gene Expression Profiling Interactive Analysis database, and Kaplan-Meier survival data to determine the association between MZT2A expression and NSCLC prognosis. Furthermore, NSCLC tissue specimens from 132 patients were evaluated with immunohistochemistry. MZT2A was overexpressed or knocked down in NSCLC cells using cDNA and siRNA, respectively. The cells were subjected to various assays and treated with the selective Akt inhibitor LY294002 or co-transfected with galectin-3-binding protein (LGALS3BP) siRNA.

Result: The levels of MZT2A mRNA and protein were upregulated in NSCLC lesions and MTZ2A expression was associated with larger tumor size, lymph node metastasis, and advanced TNM stages for NSCLC as well as poor NSCLC prognosis. MZT2A protein was also highly expressed in NSCLC cells compared to the expression in normal bronchial cells. MZT2A expression promoted NSCLC cell viability and invasion, whereas MTZ2A siRNA had the opposite effects on NSCLC cells in vitro. At the protein level, MZT2A induced Akt phosphorylation, promoting NSCLC proliferation and invasion (but the selective Akt inhibitor blocked these effects) through the upregulation of LGALS3BP via the MTZ2A MOZART2 domain, whereas LGALS3BP siRNA suppressed MTZ2A activity in NSCLC cells.

Conclusions: MZT2A exhibits oncogenic activity by activating LGALS3BP and Akt in NSCLC. Future studies will assess MTZ2A as a biomarker to predict NSCLC prognosis or as a target to control NSCLC progression.

1. Introduction

Lung cancer is the most commonly occurring cancer and the leading cause of cancer death globally, accounting for 11.6% of the 18.1 million new cases and 18.4% of the 9.6 million cancer-related deaths in 2018 [1]. Histologically, up to 85% of all lung cancer cases are non-small-cell lung cancers (NSCLCs) and the rest are small cell lung cancers [2–4]. Most NSCLCs are diagnosed at advanced stages of the disease, making curable surgery impossible. Routine chemoradiation therapy is frequently ineffective for controlling NSCLCs; thus, the 5-year survival rate of NSCLC patients is only 10–20% in most parts of the world [5, 6]. In this regard, it is necessary for researchers to better understand the molecular mechanisms of NSCLC tumorigenesis. This will enable the development and identification of novel strategies or biomarkers for the prevention and treatment of this deadly disease and facilitate early diagnosis as well as the prediction of the treatment responses and prognosis of lung cancer patients [7, 8].

Microtubules are dynamic and hollow tubular structures that form the cell cytoskeleton [9–11]. They molecularly consist of tubulin and microtubule accessory proteins and regulate biological processes such
as cell mobility and gene expression in cells [9–11]. Microtubules are nucleated and organized by microtubule organizing centers (MTOCs), specifically the centrosome. Many proteins bind to microtubules, such as the motor proteins kinesin and dynein as well as microtubule-severing proteins, which regulate the microtubule dynamics. The aberrant expression and/or activity of these proteins is associated with the development of breast [12], lung [13–15], liver [16], gastric [17, 18], and thyroid cancers [19]. Targeting microtubulin proteins and activity has been shown to be effective for the treatment of various human cancers [20, 21], although drug resistance remains an unresolved issue. The mitotic spindle organizing protein 2A (MZT2A; also known as GCP8a or FAM128A) mediates cell septation signaling during cell growth through a balance of microtubule polymerization and depolymerization at the centrosome [22–24]. MZT2A was initially identified in 2010 by Hutchins et al, who named the protein MOZART2 [22]. Teixido-Travesa et al also identified MZT2A in 2010 as a protein in the γ-tubulin ring complex (γ-TuRC) with a size of up to 20 KDa [24]. Since then, it has become evident that a lack of MZT2A expression in cells can impair the microtubule nucleation activity and slow the rate of cell proliferation [22, 24]. Thus, further exploration of the MZT2A expression and activity in NSCLCs can help clinicians effectively control NSCLC development and progression as a novel strategy for the development of biomarker and targeting therapies.

In this study, we first screened several online databases and performed bioinformatic analyses of differential MZT2A expression in these databases. We then assessed MZT2A expression in NSCLC tissue to determine the association with the prognosis of patients and also investigated the role of MZT2A in lung cancer cells in vitro.

2. Materials And Methods

2.1 Databases and data analysis

In this study, we searched The Cancer Genome Atlas (TCGA) and Gene Expression Profiling Interactive Analysis (GEPIA) databases and analyzed Kaplan-Meier survival data. We then assessed the differential MZT2A expression to determine the association with the prognosis for lung cancer. We first retrieved lung cancer data on MZT2A expression from the TCGA database, which consisted of processed level 3 RNA-seq data and the corresponding clinical information for patients, and assessed the differential MZT2A expression in NSCLCs vs. normal tissues. Next, we retrieved data from the GEPIA database and further analyzed the differential MZT2A expression in lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC) vs. normal tissues. We also performed Kaplan-Meier survival analysis to determine the association between MZT2A expression and NSCLC prognosis.

2.2 Patients and specimens

We retrospectively analyzed tissue specimens collected from 132 NSCLC patients who utilized in-patient services at the Pathology Department of the First Affiliated Hospital of China Medical University (Shenyang, China) form 2013 to 2017. All patients underwent curative surgical resection without prior chemotherapy or radiation therapy and their NSCLC was diagnosed and classified according to the TNM
classification, 8th edition [25]. Paraffin blocks of paired tumor and normal tissues were retrieved, histologically re-confirmed, and used for this study. This study was approved by the Medical Research Ethics Committee of China Medical University and informed consent was obtained from all patients.

2.3 Immunohistochemistry

Buffered formalin-fixed and embedded paraffin blocks were retrieved from the Pathology Department and cut into 3 µm-thick consecutive sections. For immunohistochemistry, the tissue sections were first deparaffinized in xylene, rehydrated in a series of ethanol solutions, and blocked with normal goat non-immune serum (SP Kit-A3/B3, MaiXin; Fuzhou, China). The sections were then incubated with an anti-MZT2A antibody at a dilution of 1:200 (Thermo, Waltham, MA, USA) or an anti-galectin-3-binding protein (LGALS3BP) antibody at a dilution of 1:300 (Cat. 10281-1-AP; Proteintech, Wuhan, China) at 4°C overnight. The next day, the sections were washed in ice-cold phosphate buffered saline (PBS) thrice and further incubated with reagents A and B (KIT9921; MaiXin) according to the manufacturer’s instructions. After color reaction, the immunostained tissue sections were mounted with neutral resin. Afterward, the staining intensity and percentage were reviewed and scored semi-quantitatively by two investigators blinded to the clinical data. The staining intensity was rated as 0, (no staining of tumor cells), 1 (weak staining), 2 (moderate staining), or 3, (strong staining), while the percentage of staining was rated as 1, (0-25% of tumor cells stained), 2 (26-50%), 3 (51%-75%), or 4 (>76%). The two scores were then multiplied to obtain the staining index (between 0 and 12). A staining index > 3 indicated high MZT2A expression, whereas a number ≤ 3 indicated low MZT2A expression.

2.4 Cell lines and culture

Human NSCLC A549, NCI-H1299, NCI-H460, NCI-H226, NCI-H292, SK-MES-1, and NCI-H661 cell lines were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and maintained (except SK-MES-1) in Roswell Park Memorial Institute medium-1640 (RPMI-1640; Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS; Clark Biosciences, Richmond, VA, USA) in a humidified incubator at 5% CO2 and 37°C. The normal human bronchial epithelial (HBE) cell line was obtained from the Cell Bank of the Chinese Academy of Sciences and cultured in Dulbecco’s Modified Eagle’s medium (DMEM) with high glucose. SK-MES-1 cells were cultured in Eagle’s Minimal Essential Medium (MEM) supplemented with 10% FBS under the same culture conditions.

2.4 Plasmid construction and cell transfection

Plasmids carrying MZT2A cDNA and vector only were obtained from GenePharma (Shanghai, China), while MZT2A-specific small-interfering RNA (si-RNA) and negative control siRNA were obtained from Riobobio (Guangzhou, China). The LGALS3BP siRNA and negative control siRNA were obtained from GenePharma. We also constructed two MZT2A mutants with alterations in the MOZART2 domain (Table 1). The MOZART2 domain was knocked out in the first mutant (MZT2A-ΔMOZART2) while retaining the antibody recognition sequence. In the second mutant (MZT2A-MOZART2), all sequences were knocked out, with the exception of the MOZART2 domain and the antibody recognition sequences. After DNA-
sequencing confirmation, the two plasmids were transfected into NSCLC cells. For cell transfection, the cells were grown and transiently transfected with the above vectors or siRNAs using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) for 48h according to the manufacturers’ protocols. To suppress the Akt signaling activity, after gene transfection for 36 h, the cell lines were treated with 10 µM LY294002 (MedChemExpress, NJ, USA) dissolved in dimethyl sulfoxide (DMSO) for 12 h. DMSO treatment was used as the negative control. The siRNA sequences targeting MZT2A or LGALS3BP and the plasmids carrying MZT2A cDNA are shown in Table 1 and Table 2, respectively.

2.5 Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

Total cellular RNA was isolated from the transient transfected cells using the Trizol reagent (TransGen Biotech, Beijing, China), quantified using a spectrophotometer (IMPLEN, Munich, Germany), and reverse transcribed into cDNA using the PrimeScript RT Master Mix kit (TAKARA, Japan) according the manufacturers’ instructions. The cDNA samples were subjected to quantitative PCR (qPCR) amplification of the MZT2A, AKT, and GAPDH mRNAs using specifically designed primers (Table 3) with the TB Green Premix EX Taq II Kit (TAKARA). The relative mRNA levels were determined using the comparative method (ΔΔCT) followed by normalization to the GAPDH level (an internal control).

2.6 Immunocytochemistry

A549 and NCI-H1299 cells were grown on coverslips and then collected and fixed in 4% paraformaldehyde for 20 min. Afterward, the cells were treated with 0.25% Triton-X100 at room temperature for 15 min, blocked in 5% bovine serum albumin (BSA) in PBS at 37°C for 2 h, and incubated with a primary antibody (Table 4) overnight at 4°C. The next day, the cells were washed with ice-cold PBS three times and incubated with a fluorescent secondary antibody (Table 4) at 37°C in the dark for 2 h. After washing with PBS thrice, the cells were stained with 4',6-diamidino-2-phenylindole (DAPI, Solarbio, Beijing, China) for 10 min, mounted with glycerin, and reviewed and scored under a fluorescence microscope (Olympus, Tokyo, Japan).

2.7 Western blot

Transiently transfected cells were washed with ice-cold PBS and lysed using a lysis buffer (Beyotime, Shanghai, China) containing a protease-inhibitor cocktail (Beyotime) and a phosphatase-inhibitor cocktail (MedChemExpress). After quantitation using the bicinchoninic acid (BCA) protein assay, equal amounts of protein samples were separated in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto polyvinylidene difluoride membranes (Invitrogen). For Western blotting, the membranes were blocked with 5% skimmed dry milk solution in Tris-based saline-Tween 20 (TBS-T) at room temperature for 2 h and incubated with a primary antibody (Table 4) in 2%BSA in TBS-T at 4°C overnight. The next day, the membranes were washed with TBS-T thrice and incubated at 37°C with horseradish peroxidase-conjugated anti-mouse/rabbit IgG at a dilution of 1:20000 (Zsbio, Beijing, China) for 2 h. After washing again with TBS-T, the membranes were incubated with the enhanced chemiluminescence reagent (Beyotime) to visualize the positive protein bands captured using an
enhanced chemiluminescence device (Thermo Fisher Scientific, Waltham, MA USA). The results were quantified using ImageLab 3.0 software (Bio-Rad, Hercules, CA, USA).

**2.8 Cell viability and invasion assays**

Changes in the cell viability after gene manipulation were evaluated with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) kit (Beyotime, Shanghai, China). In brief, gene-transfected H1299 and A549 cells were re-plated into 96-well plates in triplicate with 100 μL growth medium and cultured for 0, 24, 48, 72, or 96 h. H1299 cells were re-plated with 5×10⁴ cells/well while A549 cells were re-plated with 8×10⁴ cells/well. At the end of each experiment, 10 μL MTT reagent was added to the cell culture and the cells were cultured for an additional 4 h. The optical absorbance rate was measured using a microplate spectrophotometer (Thermo) at 450 nm and the percentage compared to the control was calculated.

Changes in cell invasion capacity were assessed using 24-well Transwell chambers containing inserts with an 8-μM pore size (Costar, Washington, DC, USA) and coated with 100 μL Matrigel (a 1:9 dilution; BD Biosciences, San Jose, CA, USA). In particular, gene-transfected A549 and H1299 cells were suspended in RPMI-1640 without serum at 8 × 10⁴ cells/mL for A549 cells and 5 × 10⁴ cells/mL for H1299 cells. Hundred microliters of each cell solution was added to the upper chamber in triplicate, while the lower chamber was filled with 600 μL RPMI-1640 containing 20% FBS. The plates were then incubated in a humidified incubator with 5% CO₂ at 37°C for 24 h. At the end of the incubation period, the cells remaining in the upper chamber were removed with a cotton swab. The cells that migrated to the other side of the chambers were fixed with 4% paraformaldehyde for 10 min and stained with 0.1% crystal violet solution for 10 min. The number of invading cells was counted in five randomly selected fields under a light microscope (Leica, Wetzlar, Germany).

**2.9 Co-immunoprecipitation (Co-IP)-Western blot**

The gene-transfected A549 and H1299 cells with or without treatment were collected through an ice-cold PBS wash and lysed in a lysis buffer (see Western blot part). After quantitation, 2 mg of each protein sample was incubated with Protein A+G Agarose (Beyotime) at 4°C for 150 min. The samples were washed with TBS-T through centrifugation at 1000 rpm in a clinical centrifuge and incubated with anti-Myc-tag antibody or anti-IgG (as a control) (Table 4) at 4°C overnight on a shaking platform. The next day, Protein A+G agarose beads were added to the mixture to capture the binding antibody complex at 4°C for 6 h. After centrifugation, the supernatants were collected and analyzed using Western blot.

**2.10 Statistical analysis**

The data were summarized as the mean ± standard deviation (SD) and analyzed statistically with the one-way analysis of variance (ANOVA) test to compare the differences among the groups. The Pearson correlation test was performed to determine the correlation between MZT2A and LGALS3BP levels in NSCLC tissue samples. All statistical analyses were performed using the SPSS software (version 22.0; SPSS, Chicago, IL, USA). A p value equal to or less than 0.05 was considered statistically significant.
3. Results

3.1 Upregulation of MZT2A expression in NSCLCs and association with poor NSCLC prognosis

In this study, we first assessed MZT2A expression in NSCLCs using TCGA data. The initial analysis revealed the differential expression of MZT2A mRNA between NSCLCs and normal tissues ($p < 0.01$) and confirmed the data in the GEPIA database, which showed that MZT2A expression was higher in LUAD and LUSC than in normal tissues ($p < 0.01$). Our Kaplan-Meier analysis using Kaplan-Meier data revealed that MZT2A expression was associated with poor prognosis for NSCLC patients (Fig. 1A and 1B).

We next collected our own samples from a cohort of 132 NSCLC patients and analyzed the MZT2A expression immunohistochemically. Our data further confirmed the TCGA and GEPIA data showing that the MZT2A protein was mainly localized in the cytoplasm, although a few cell nuclei were positive for MZT2A protein and MZT2A expression was higher in NSCLC tissues than in normal lung and bronchial tissues (Fig. 1C). MZT2A expression was associated with the tumor size, lymph node metastasis, and advanced TNM stages (Table 5).

3.2 MZT2A promotion of NSCLC cell malignant behaviors in vitro

We assessed the levels of MZT2A protein in different lung cancer cell lines and found that the expression of MZT2A protein was higher in cancer cell lines than in normal cells, with the exception of NCI-H460 cells (Fig. 1D). For our subsequent experiments, we selected the A549 and H1299 cell lines, both of which showed a moderate level of MZT2A expression. Our immunocytochemical staining further confirmed the cytoplasmic localization of MZT2A in the centrosomes and cytoplasm, although some nuclei were also positive for MZT2A in A549 and NCI-H1299 cells (Fig. 1E).

We further explored the effect of MZT2A overexpression and knockdown on the regulation of NSCLC cell viability, invasion, and gene expression. We first confirmed MZT2A overexpression and knockdown with MZT2A cDNA and siRNA transfection in A549 and H1299 cells, detected using Western blot and qRT-PCR (Fig. 2A and 2B). Afterward, we performed a cell viability MTT assay and found that the cell viability was reduced after the knockdown of MZT2A expression in both A549 and H1299 cell lines, whereas tumor cell viability was upregulated following MTZ2A overexpression ($p < 0.05$; Fig. 2C). Furthermore, the tumor cell invasion capacity was suppressed after the knockdown of MTZ2A expression, whereas MTZ2A overexpression enhanced the tumor cell invasion capacity compared to that of control cells ($p < 0.05$; Fig. 2D). In addition, the levels of cyclinD1, CDK4, CDK6, RhoA, and Vimentin expression were downregulated by MZT2A siRNA, whereas MZT2A cDNA had the opposite effect on the expression of these proteins compared with the levels in the control cells ($p < 0.05$; Fig. 2E).

3.3 MZT2A regulation of NSCLC cell malignant behaviors via Akt phosphorylation

After screening various signal transduction molecules, we speculated that Akt signaling might modulate the MZT2A-induced malignant behaviors of A549 and H1299 cells. We found that the level of phosphorylated Akt protein was reduced after MTZ2A siRNA transfection, whereas MTZ2A cDNA had the
opposite effect on Akt phosphorylation ($p < 0.05$; Fig. 3A). However, the total Akt protein and mRNA levels showed no significant changes in cells transfected with MTZ2A siRNA or cDNA ($p > 0.05$; Fig. 3A and 3B).

We added treatment with an inhibitor (LY294002) to block Akt signaling in our experimental setting and found that LY294002 treatment suppressed Akt phosphorylation but did not alter the total Akt level (Fig. 3C). We also found that the degree of increase in the phosphorylated-Akt level after MZT2A cDNA transfection was reduced by LY294002 treatment, but the total Akt protein level barely changed ($p < 0.05$; Fig. 3C). The promoting effect of MZT2A overexpression on NSCLC cell viability and invasion was also inhibited by LY294002 ($p < 0.05$; Fig. 3D and 3E).

3.4 MZT2A activation of Akt signaling via galectin-3-binding protein (LGALS3BP)

To further explore the underlying molecular events, we searched various biomedical databases and retrieved data suggesting that LGALS3BP is linked to MZT2A activation of the Akt protein. We then performed Co-IP-Western blot analysis and confirmed the interaction between MZT2A and LGALS3BP in A549 and H1299 cells (Fig. 4A). Our Pearson correlation analysis of immunohistochemical data on MTZ2A and LGALS3BP also confirmed this relationship ($p < 0.05$; Fig 4B). Co-transfection of MZT2A cDNA and LGALS3BP siRNA into A549 and H1299 cells showed that compared to the MZT2A cDNA alone, LGALAS3BP siRNA inhibited the promoting effect of MZT2A on the level of Akt phosphorylation, whereas LGALS3BP siRNA alone did not alter the level of MZT2A protein ($p < 0.05$; Fig. 4C). The level of LGALS3BP protein was upregulated after MZT2A cDNA transfection in NSCLC cells ($p < 0.05$; Fig. 4C). In addition, the promoting effects of MZT2A on NSCLC cell viability and invasion were also reduced by LGALS3BP siRNA ($p < 0.05$; Fig. 4D and 4E).

3.5 MZT2A activation of LGALS3BP and Akt phosphorylation via the MOZART2 domain

To further investigate the molecular mechanism underlying the effects of MZT2A and LGALS3BP in NSCLC cells, we constructed two MZT2A mutant vectors (Fig. 5A and 5B) and performed Co-IP-Western blot assays. Our data showed positive results in groups transfected with MZT2A-WT and MZT2A-MOZART2 vectors (Fig. 5C and 5D). Specifically, without the MOZART2 domain, MZT2A could not bind to or regulate LGALS3BP expression in A549 and H1299 cells. Our Western blot data showed that only with an intact MOZART2 domain was MZT2A able to induce LGALS3BP expression and Akt phosphorylation ($p < 0.05$; Fig. 5E and 5F) as well as promote A549 and H1299 cell viability and invasion ($p < 0.05$; Fig. 5G and 5H).

4. Discussion

In the current study, we identified differential MZT2A expression in NSCLC tissues vs. normal lung tissues and the association between MZT2A expression and NSCLC prognosis. We then overexpressed or knocked down MZT2A expression in NSCLC cells using MZT2A cDNA and siRNA, respectively, to assess the oncogenic activity of MTZ2A in NSCLCs. We found that both MZT2A mRNA and protein were upregulated in NSCLC tissues, which was associated with larger NSCLC size, lymph node metastasis,
advanced TNM stages of NSCLCs, and poor NSCLC prognosis. In vitro, MZT2A protein was highly expressed in all NSCLC cell lines except one (NCI-H460) compared to the expression in normal bronchial cells. MZT2A overexpression promoted NSCLC cell viability and invasion, whereas MTZ2A siRNA transfection had the opposite effects. Moreover, MZT2A induced Akt phosphorylation to promote NSCLC proliferation and invasion, and these effects were blocked by the selective Akt inhibitor LY294002. MTZ2A also upregulated LGALS3BP expression through the MOZART2 domain, whereas LGALS3BP siRNA suppressed MTZ2A-induced Akt phosphorylation in NSCLC cells. In conclusion, MZT2A was overexpressed in NSCLC tissues, which was associated with poor NSCLC prognosis. MZT2A overexpression promoted NSCLC cell viability and invasion through LGALS3BP upregulation and Akt phosphorylation, indicating that MZT2A possesses oncogenic activities in NSCLCs. Future studies should further evaluate MTZ2A as a biomarker for the prediction of NSCLC prognosis or as a target for the control of NSCLCs.

Previous studies have shown that MZT2A can induce the proliferation of various types of human cancer cells [22, 24]. It has been demonstrated that microtubules can regulate tumor cell metastasis and invasion [26]. As a component of microtubules, we propose that MZT2A not only functions to regulate the cell cycle and cell proliferation as reported in the literature [24, 27], but also affects the mobility of NSCLC cells (our current data). The changes caused by MZT2A in the malignant behaviors of NSCLC cell illustrated in our current study were due to the induction of Akt phosphorylation. Indeed, a high level of Akt phosphorylation has been detected in various human cancers and plays a role in many different biological activities during the transformation of normal cells to cancerous cells [28–30]. For example, Akt can promote cell cycle progression [31], and cell proliferation [32] as well as cell survival [33]. Akt regulates lysosomal biogenesis, autophagy, and angiogenesis, all of which are important in human carcinogenesis [34, 35]. Akt activation is also associated with many malignancies [36, 37]. Previous studies have shown that Akt phosphorylation is increased in many tumors, including NSCLCs [38, 39]. Our current study further confirmed the role of Akt in the promotion of NSCLC cell proliferation and invasion. We demonstrated that MZT2A could promote Akt phosphorylation; however, MZT2A is a protein in the centrosome with no kinase activity, suggesting that the MZT2A activation of Akt is indirect. Thus, we speculated that there might be another molecule that serves as a bridge (link) between MZT2A and Akt to induce Akt phosphorylation. We searched protein-protein interaction databases and identified LGALS3BP as a potential linker. Indeed, our Co-IP-Western blot and immunohistochemical data supported this speculation by showing a physical interaction between MZT2A and LGALS3BP protein. Thereafter, we found that LGALS3BP was able to upregulate Akt signaling activity by increasing Akt phosphorylation. A literature search uncovered two theories about the specific mechanism employed by LGALS3BP for the regulation of Akt phosphorylation. One theory is that LGALS3BP induces Akt phosphorylation at the Ser473 site through integrins [40–42]. The other theory is that LGALS3BP binds to galectin 3 to activate phosphoinositide-dependent kinase 1 and in turn upregulates the level of Akt phosphorylation at the Thr308 site [40–42]. In our current study, we detected MZT2A activation of Akt phosphorylation at the Thr308 site. However, our results indicated that the Thr308 site phosphorylation induced by MZT2A was not stable, whereas the change caused by MZT2A at the Ser473 phosphorylation site appeared steady.
Indeed, MZT2A is a protein with a small molecular weight and previous studies focused on the role of MZT2A in cell mitosis, especially the formation of the spindle [22, 24, 27]. In this regard, functional descriptions of the unique MZT2A MOZART2 domain were also focused on this process. Though MZT2A depletion has not been reported to affect the γ-TuRC assembly, it interferes with γ-TuRC recruitment and microtubule nucleation in the interphase centrosomes, but does not disrupt the general centrosome structure, indicating that MZT2A plays a role in the organization of the interphase microtubule network [22]. In our current study, we mutated the MOZART2 domain and observed that the mutation disrupted Akt phosphorylation as well as NSCLC cell viability and invasion capacity, indicating that the MOZART2 domain is necessary for MZT2A to function in NSCLC cells.

However, our current study is merely a proof of principle and several limitations exist; for example, most of our data are descriptive and the study lacks precise mechanism experiments. NSCLC development is a multi-factorial process. The upregulation of MZT2A expression may also modulate the expression and activity of other proteins in NSCLC cells to promote NSCLC development and progression. However, our current data only show one example of MZT2A action in NSCLC cells. In future studies, we will explore the cause of MZT2A overexpression in NSCLCs and the underlying molecular mechanisms of MZT2A-induced NSCLC development and progression.

In conclusion, our current data, for the first time, demonstrated that the overexpression of MZT2A mRNA and protein was associated with NSCLC progression and poor prognosis. In vitro, MZT2A promoted NSCLC malignant behaviors by increasing Akt phosphorylation via LGALS3BP in NSCLC cells. We also identified the importance of the MOZART2 domain for a functional MZT2A protein in NSCLC cells.

**Abbreviations**

DMSO: Dimethyl sulfoxide; FBS: Fetal Bovine serum; GEPIA: Gene expression profiling interactive analysis; LGALS3BP: Galectin 3 Binding Protein; LUAD: lung adenocarcinoma; LUSC: lung squamous cell carcinoma; MEM: Modified Eagle's medium; MOZART2: MZT2A: Mitotic spindle organizing protein 2A; NSCLC: non-small cell lung cancer; PBS: Phosphate-buffered saline; siRNA: Small-interfering RNA; TCGA: The cancer genome atlas;

**Declarations**

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**Availability of data and materials**

All data generated or analyzed during this study are included in this article. Further details are available from the corresponding author upon request.
Conflict of interest statement

The authors declare that there is no conflict of interest in this work.

Consent for publish

Not applicable

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Author contribution

Huanxi Wang, Hongjiu Ren, and Xueshan Qiu conceived and designed the experiments; Huanxi Wang, Zifang Zou, and Yu Cheng prepared the manuscript; Huanxi Wang, Xizi Jiang, Yujiao Hu, Zongang Liu, Jiameng Zhang and Qiongzi Wang performed the experiments; Yao Zhang and Hongbo Su analyzed the data.

Ethics approval and consent to participate

This study was approved by the Medical Research Ethics Committee of China Medical University and informed consent was obtained from all patients.

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Tables

Table 1. Plasmid carrying wild type and mutated MZT2A cDNA
| MZT2A cDNA | Targeted Gene sequences |
|------------|------------------------|
| MZT2A-WT   | 5'-TCCGGAATGGGGCGCCAGGGCGTAGGTCCTTGCCCACGTGGGCAGCGCTGCCCTCTGAGGCTGGGGAGTATTGAGCCAGGAGAGAGAGAGGAGGAGAGGATCCAGGGGAGCTGTACGAGCTGGCTCAGGCGGCGGGCGGCGGTAAGGGCTGCCCACGTCGAGCGTGCCCGAGACCCGAGGGAGAGACAAAGGCAGCGCTGCCCTCGGGGGAGTATTGCCCTGGCGGAACGCAGCAACCACGAGGGATCCAAGCCAGAGGATGCCACGCCAGCCCAGCGCTACCAGGCTGCCCAAGGGGGGCGGGCCTGGGAAGAGCCCTACGCAGGGCAGCACCAGATCT-3' |
| MZT2A-ΔMOZART2 | 5'-TCCGGAATGGGGCGCCAGGGCGTAGGTCCTTGCCCACGTGGGCAGCGCTGCCCTCTGAGGCTGGGGAGTATTGAGCCAGGAGAGAGAGGAGGAGGATCCAGGGGAGCTGTACGAGCTGGCTCAGGCGGCGGGCGGCGGTAAGGGCTGCCCACGTCGAGCGTGCCCGAGACCCGAGGGAGAGACAAAGGCAGCGCTGCCCTCGGGGGAGTATTGCCCTGGCGGAACGCAGCAACCACGAGGGATCCAAGCCAGAGGATGCCACGCCAGCCCAGCGCTACCAGGCTGCCCAAGGGGGGCGGGCCTGGGAAGAGCCCTACGCAGGGCAGCACCAGATCT-3' |
| MZT2A-MOZART2 | 5'-TCCGGAATGGGGCGCCAGGGCGTAGGTCCTTGCCCACGTGGGCAGCGCTGCCCTCTGAGGCTGGGGAGTATTGAGCCAGGAGAGAGAGGAGGAGGATCCAGGGGAGCTGTACGAGCTGGCTCAGGCGGCGGGCGGCGGTAAGGGCTGCCCACGTCGAGCGTGCCCGAGACCCGAGGGAGAGACAAAGGCAGCGCTGCCCTCGGGGGAGTATTGCCCTGGCGGAACGCAGCAACCACGAGGGATCCAAGCCAGAGGATGCCACGCCAGCCCAGCGCTACCAGGCTGCCCAAGGGGGGCGGGCCTGGGAAGAGCCCTACGCAGGGCAGCACCAGATCT-3' |
The bold letters indicate the domain sequences for MOZART2 and the underlined letters are mutated.

**Table 2. MZT2A, LGALS3BP, and negative control siRNA sequences**

| siRNA      | Sense             | Antisense                     |
|------------|-------------------|-------------------------------|
| MZT2A      | 5'-UGGCGGAACGCAGCAACCA-3' | 5'-UGGUUGCUGCUGCUCCGCA-3'  |
| LGALS3BP   | 5'-GCGUGGAAACGCAUGUGACAUU-3' | 5'-AUGUCAACCAUCGUUCAGGT-3'  |
| Negative control | 5'-UUCUCCGAACGUCUGACTTT-3' | 5'-ACGUGACACGUCGGAGAATT-3' |

**Table 3. PCR primer sequences**

| Gene   | Primer sequences                      | Tm (°C) |
|--------|--------------------------------------|---------|
| MZT2A  | 5’-TTTCTCCAGATGCTCAAGTCC-3’          | 55.4    |
|        | 5’-TTTTGTCTCTCCCTCGGTC-3’            |         |
| AKT    | 5’-TCACTCCATCAAGATGTCAAGCA-3’        | 55.6    |
|        | 5’-TCACTGACGATGCTTCTTC-3’            |         |
| GAPDH  | 5’-AGGTGGGCTTGGAACTGGTTT-3’          | 58      |
|        | 5’-TCTAGACCATGTTGTTAGT-3’            |         |

**Table 4. Antibodies used in immunocytochemistry and Western blot**
| Protein name | Manufacturer     | Host    | Dilution                          |
|--------------|------------------|---------|-----------------------------------|
| MZT2A        | Thermo           | Rabbit  | Immunocytochemistry, 1:50 Western blot, 1:500 |
| GAPDH        | Proteintech      | Mouse   | 1:10000                           |
| RhoA         | Cell Signaling Technology | Rabbit | 1:1000                           |
| Cyclin D1    | Cell Signaling Technology | Rabbit | 1:1000                           |
| CDK4         | Cell Signaling Technology | Rabbit | 1:1000                           |
| CDK6         | Cell Signaling Technology | Rabbit | 1:1000                           |
| Akt          | Proteintech      | Rabbit  | 1:500                            |
| P-Akt        | Proteintech      | Mouse   | 1:500                            |
| LGALS3BP     | Proteintech      | Rabbit  | 1:500                            |
| Vimentin     | Proteintech      | Rabbit  | 1:500                            |
| Tag-myc      | TransGen         | Mouse   | 1:500                            |
| Anti-mouse IgG | Zsbio           | Goat    | 1:20000                          |
| Anti-rabbit  | Zsbio            | Goat    | 1:20000                          |
| Fluorescent Anti-rabbit-TRITC | Zsbio | Goat    | 1:100                            |

Table 5. Association of MZT2A expression with clinicopathological data from patients
| Variables                      | $n$ | MZT2A expression | $p$ value |
|-------------------------------|-----|------------------|-----------|
| Age (yrs.)                    |     |                  |           |
| $\leq 60$                     | 59  | 34               | 25        |
| $< 60$                        | 73  | 39               | 34        | 0.38     |
| Gender                        |     |                  |           |
| Male                          | 94  | 59               | 35        |
| Female                        | 38  | 14               | 24        | 0.20     |
| Histological type             |     |                  |           |
| Squamous cell carcinoma       | 62  | 43               | 19        |
| Adenocarcinoma                | 70  | 30               | 40        | 0.12     |
| Tumor differentiation         |     |                  |           |
| Well                          | 69  | 39               | 30        |
| Poor                          | 63  | 35               | 28        | 0.52     |
| Tumor size (cm)               |     |                  |           |
| $< 3$                         | 63  | 25               | 38        |
| $\geq 3$                      | 69  | 48               | 21        | $< 0.01$ |
| Lymph node metastasis         |     |                  |           |
| Negative                      | 75  | 31               | 44        |
| Positive                      | 57  | 42               | 15        | $< 0.01$ |
| TNM stage                     |     |                  |           |
| I                             | 63  | 26               | 37        |
| II-III                        | 69  | 47               | 22        | $< 0.01$ |

**Figures**
Figure 1

MZT2A overexpression and association with poor NSCLC prognosis. A, Differential MZT2A expression in NSCLCs using TCGA data (i) and GEPIA data (ii). LUAD, lung adenocarcinoma and LUSC, lung squamous cell carcinoma. B, Kaplan-Meier survival analysis. High MZT2A expression is associated with poor NSCLC prognosis. C, Immunohistochemistry. MZT2A expression in our cohort of 132 NSCLC cases was analyzed. i, Weak MZT2A expression in normal bronchial epithelium. ii, strong staining of MZT2A in mitosis-stage cells. iii-iv, MZT2A expression in lung squamous cell carcinoma and adenocarcinoma cells. D, Western blot. NSCLC cell lines and HBE cells were grown and harvested for Western blot analysis. The data showed that MZT2A expression was highest in H292 cells and lowest expressed in H460 cells. The graph is the quantified data for the Western blots. E, Immunofluorescence. NSCLC A549 and H1299 cells were grown and immunostained with an MZT2A antibody. The data showed that MZT2A was mainly localized in the cytoplasm and a few in the nuclei.
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MZT2A promotion of NSCLC cell viability and invasion as well as regulation of protein expression in A549 and H1299 cells. A, Western blot. NSCLC A549 and H1299 cells were grown, transfected with MZT2A cDNA or siRNA, and subjected to Western blot. The data showed that MZT2A cDNA or siRNA transfection respectively up- and down-regulated MZT2A expression. The graphs are the quantified data. B, qRT-PCR. NSCLC A549 and H1299 cells were grown, transfected with MZT2A cDNA or siRNA, and...
subjected to qRT-PCR. C, Cell viability MTT assay. NSCLC A549 and H1299 cells were grown, transfected with MZT2A cDNA or siRNA, and subjected to cell viability assays. The data showed that the up- or down-regulation of MZT2A expression promoted or reduced NSCLC cell viability. D, Transwell invasion assay. NSCLC A549 and H1299 cells were grown, transfected with MZT2A cDNA or siRNA, and subjected to Transwell assays. The graphs are the quantified data. E, Western blot. NSCLC A549 and H1299 cells were grown, transfected with MZT2A cDNA or siRNA, and subjected to Western blot analysis of the proteins associated with cell proliferation and invasion. The graphs are the quantified data.
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MZT2A promotion of NSCLC cell viability and invasion as well as regulation of protein expression in A549 and H1299 cells. A, Western blot. NSCLC A549 and H1299 cells were grown, transfected with MZT2A cDNA or siRNA, and subjected to Western blot. The data showed that MZT2A cDNA or siRNA transfection respectively up- and down-regulated MZT2A expression. The graphs are the quantified data. B, qRT-PCR. NSCLC A549 and H1299 cells were grown, transfected with MZT2A cDNA or siRNA, and subjected to qRT-PCR. C, Cell viability MTT assay. NSCLC A549 and H1299 cells were grown, transfected with MZT2A cDNA or siRNA, and subjected to cell viability assays. The data showed that the up- or down-regulation of MZT2A expression promoted or reduced NSCLC cell viability. D, Transwell invasion assay. NSCLC A549 and H1299 cells were grown, transfected with MZT2A cDNA or siRNA, and subjected to Transwell assays. The graphs are the quantified data. E, Western blot. NSCLC A549 and H1299 cells were grown, transfected with MZT2A cDNA or siRNA, and subjected to Western blot analysis of the proteins associated with cell proliferation and invasion. The graphs are the quantified data.
Figure 3

MZT2A regulation of Akt phosphorylation via LGALS3BP expression. A, Western blot. NSCLC A549 and H1299 cells were grown, transfected with MZT2A cDNA or siRNA, and subjected to Western blot. The graphs are the quantified data. B, qRT-PCR. NSCLC A549 and H1299 cells were grown, transfected with MZT2A cDNA or siRNA, and subjected to qRT-PCR. C, Western blot. A549 and H1299 cells were grown, transfected with MZT2A cDNA, treated with or without the selective Akt inhibitor LY294002, and subjected to Western blot analysis. The graphs are the quantified data. D, Cell viability MTT assay: A549 and H1299
cells were grown, transfected with MZT2A cDNA, treated with or without the selective Akt inhibitor LY294002, and subjected to MTT assays. E, Transwell assay. A549 and H1299 cells were grown, transfected with MZT2A cDNA, treated with or without the selective Akt inhibitor LY294002, and subjected to Transwell assays. The graphs are the quantified data.

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Figure 4

MZT2A-activated Akt signal transduction via LGALS3BP. A, Co-IP-Western blot. A549 and H1299 cells were grown and subjected to Co-IP-Western blot to detect the intact interaction between MZT2A and LGALS3BP proteins B, Immunohistochemistry and Pearson correlation analysis. NSCLC tissue sections were immunostained with an anti-MZT2A or LGALS3BP antibody and the expression of MZT2A and LGALS3BP protein were scored as high or low. The tissue sections were subjected to Pearson correlation
analysis (shown in the graph; p < 0.05). C, Western blot. A549 and H1299 cells were grown, transfected with MZT2A cDNA and/or LGALS3BP siRNA, and subjected to Western blot analysis. D, Cell viability MTT assay. A549 and H1299 cells were grown, transfected with MZT2A cDNA and/or LGALS3BP siRNA, and subjected to MTT assays. E, Transwell assay. A549 and H1299 cells were grown, transfected with MZT2A cDNA and/or LGALS3BP siRNA, and subjected to Transwell assays. The graph is the quantified data.
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Figure 5

The MZT2A functional domain MOZART2 mediates MZT2A functions in the regulation of Akt phosphorylation. A, Illustration of our creation of two mutated MZT2A MOZART2 domain constructs. ARS: antibody recognition sequences B, Western blot. A549 and H1299 cells were grown, transfected with the two mutated MZT2A cDNAs, and subjected to Western blot. C and D, Co-IP-Western blot. A549 and H1299 cells were grown, transfected with the two mutated MZT2A cDNA, and subjected to Co-IP-Western blot. E and F, Western blot. A549 and H1299 cells were grown, transfected with the two mutated MZT2A
cDNAs, and subjected to Co-IP-Western blot. G, Transwell assay. A549 and H1299 cells were grown, transfected with the two mutated MZT2A cDNAs, and subjected to Transwell assays. The graphs are the quantified data. H, Cell viability MTT assay. A549 and H1299 cells were grown, transfected with the two mutated MZT2A cDNAs, and subjected to MTT assays.

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