

Mucosa-associated lymphoid tissue lymphoma translocation protein 1 inhibitor, MI-2, attenuates non-small cell lung cancer cell proliferation, migration and invasion, and promotes apoptosis by suppressing the JNK/c-JUN pathway

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Abstract. Mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) inhibitors are effective in attenuating the progression of several types of cancer. However, their role in lung cancer requires further investigation. Therefore, the present study aimed to explore the effect of the MALT1 inhibitor, MI-2, on the behavior of non-small cell lung cancer (NSCLC) cells and to uncover their possible underlying mechanism of action. The mRNA and protein expression levels of MALT1 were detected in the human normal lung epithelial cell line BEAS-2B, and the NSCLC cell lines, NCI-H1299, NCI-H1650, HCC827, A549 and NCI-H23. Subsequently, NCI-H1650 and A549 cells were treated with MI-2. Additionally, NCI-H1650 and A549 cells were co-treated with anisomycin, a c-JUN N-terminal kinase (JNK) pathway activator, with or without MI-2. The results illustrated that the mRNA and protein expression levels of MALT1 were significantly increased in NCI-H1299, NCI-H1650, A549 and NCI-H23 cells compared with those in BEAS-2B cells. Treatment of NCI-H1650 and A549 cells with MI-2 for 72 h reduced the optical density value as determined using the Cell Counting Kit-8 assay. Consistently, the 5-ethynyl-2'-deoxyuridine assay also showed that proliferation was reduced in MI-2-treated NSCLC cells. In addition, MI-2 downregulated B-cell lymphoma 2 (BCL2), and enhanced BCL2-associated X-protein expression and apoptotic rate in NCI-H1650 and A549 cells. These findings indicated that MI-2 could inhibit NCI-H1650 and A549 cell proliferation and promote apoptosis.

Furthermore, treatment of cells with MI-2 only attenuated the migration and invasion of NCI-H1650 cells. Notably, MI-2 decreased the expression levels of phosphorylated (p)-JNK and p-c-JUN in NCI-H1650 and A549 cells, thus suggesting that MI-2 could suppress the JNK/c-JUN signaling pathway. However, NSCLC cell co-treatment with anisomycin (JNK pathway activator) reversed the effect of MI-2 on the proliferation, apoptosis and activation of the JNK/c-JUN pathway in NCI-H1650 and A549 cells. In conclusion, the present study demonstrated that the MALT1 inhibitor, MI-2, could suppress NSCLC cell proliferation, migration and invasion, and induce apoptosis via inactivating the JNK/c-JUN pathway.

Introduction

Non-small cell lung cancer (NSCLC) accounts for ~85% of all diagnosed cases of lung cancer, which is a predominant cause of cancer-related deaths worldwide (1). Currently, the treatment approaches for NSCLC include surgery, chemotherapy, radiotherapy, immunotherapy and targeted therapy, which have achieved notable advances (2). However, NSCLC remains an incurable disease and a proportion of patients with NSCLC cannot benefit from the aforementioned treatment strategies, thus resulting in disease progression and severely affecting patient prognosis (3-5). In addition, the 5-year survival rate in patients with NSCLC, which ranges between 20 and 50%, remains poor (6-8). Therefore, exploring potential pharmacological agents to attenuate NSCLC progression to improve patient prognosis is of great importance.

Mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) is a type of paracaspase, which is associated with the pathogenesis and progression of several types of cancer, including lung cancer (9,10). A previous study indicated that MALT1 silencing can inhibit the proliferation, migration, invasion and tumor-formation abilities of hepatocellular carcinoma (HCC) cells via hindering the nuclear factor- κ B (NF- κ B) pathway (11). Furthermore, another study showed that MALT1 enhances the proliferation and colony-forming abilities, and inhibits the apoptosis of prostate cancer cells, possibly due to its regulation of the NF- κ B, Wnt/ β -catenin and transforming growth factor β pathways (12). Additionally, a study illustrated that biperiden,

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a potent MALT1 inhibitor, inhibits the proliferation and accelerates the apoptosis of pancreatic ductal adenocarcinoma cells (13). In terms of lung cancer, a previous study demonstrated that MALT1 knockdown diminishes A431 and HCC827 cell migration and motility (14). Furthermore, as an integral subunit of the caspase recruitment domain family member 11/B-cell lymphoma (BCL)10/MALT1 (CBM) complex, MALT1 is a key activator of the NF- κ B pathway (9). Activation of the NF- κ B pathway can facilitate inflammation, and cancer cell proliferation and metastasis, which may accelerate NSCLC progression (15). Therefore, it has been hypothesized that MALT1 inhibitors may induce a suppressive effect on NSCLC. MI-2 is a strong MALT1 inhibitor that works by directly binding to MALT1 and inhibiting its protease function, and it is effective in slowing the progression of several types of cancer, such as glioblastoma multiforme and chronic lymphocytic leukemia (16–18). However, the effect of MI-2 on NSCLC is unclear and should be further explored.

The present study aimed to investigate the effect of a MALT1 inhibitor, namely MI-2, on the behavior of NSCLC cells, as well as its underlying mechanism of action.

Materials and methods

Cell culture. The BEAS-2B human normal lung epithelial cell line was provided by Beyotime Institute of Biotechnology, and the cells were cultured in Dulbecco's modified Eagle's medium (Beyotime Institute of Biotechnology) supplemented with 10% fetal bovine serum (FBS; HyClone; Cytiva). The NSCLC cell lines, NCI-H1299, NCI-H1650, HCC827, A549 and NCI-H23, were obtained from iCell Bioscience, Inc. NCI-H1299, H1650, HCC827 and NCI-H23 cells were maintained in RPMI-1640 medium (iCell Bioscience, Inc.), and A549 cells were maintained in Ham's F-12K medium (Procell Life Science & Technology Co., Ltd.). All media were supplemented with 10% FBS. All cells were grown in media supplemented with 1% penicillin/streptomycin solution (iCell Bioscience, Inc.) at 37°C in an incubator containing 5% CO₂.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The mRNA expression levels of MALT1 were detected by RT-qPCR. Briefly, BEAS-2B and NSCLC cells were cultured for 24 h and RNA was then extracted using the MolPure[®] Cell RNA Kit (Shanghai Yeasen Biotechnology Co., Ltd.). RT-qPCR was performed using the HiScript II One Step RT-qPCR Kit (Vazyme Biotech Co., Ltd.), according to the manufacturer's instructions. The thermocycling conditions were as follows: 55°C for 20 min, 1 cycle; 94°C for 3 min, 1 cycle; 94°C for 30 sec, 61°C for 30 sec, 72°C for 30 sec, 35 cycles; 72°C for 5 min, 1 cycle. The 2^{- $\Delta\Delta$ C_t} method was utilized to quantify mRNA expression levels (19). The specific primer sequences (5'-3') used were as follows: MALT1, forward TCTTGGCTGGACAGTTTGTGA and reverse, GCTCTCTGGGATGTCGCAA; and GAPDH, forward CCATCA CCATCTCCAGGAG and reverse CCTGCTTACCACCT TCTTG.

Assessment of the sensitivity of NSCLC cells to MI-2. The sensitivity of NCI-H1650 and A549 cells to MI-2 (MedChemExpress), a MALT1 inhibitor, was assessed using

the Cell Counting Kit-8 (CCK-8) assay (MedChemExpress). Briefly, cells were seeded into a 96-well plate at a density of 3x10³ cells/well and were then cultured in medium supplemented with 0, 0.25, 0.5, 1, 2, 4 or 8 μ mol/l MI-2 for 24 h at 37°C. Subsequently, cells were incubated with 10 μ l CCK-8 reagent for an additional 2 h. Cell viability was calculated by measuring the optical density (OD) in each well using the iMark microplate reader (Bio-Rad Laboratories, Inc.). Subsequently, the half-maximal inhibitory concentration (IC₅₀) of MI-2 in NCI-H1650 and A549 cells was calculated using GraphPad Prism 9.0 (Dotmatics).

Cell proliferation assay. Cell proliferation was assessed using CCK-8 and 5-ethynyl-2'-deoxyuridine (EdU) staining assays. Briefly, NCI-H1650 and A549 cells were cultured and stimulated with 1 or 2 μ mol/l MI-2, respectively, based on the IC₅₀ value. A total of 0, 24, 48 and 72 h after stimulation at 37°C, the cells were supplemented with CCK-8 reagent and the OD value was measured as aforementioned. In addition, EdU staining was performed using the BeyoClick[™] EdU-488 Kit (Beyotime Institute of Biotechnology). Briefly, cells were stimulated with 1 or 2 μ mol/l MI-2 for 24 h at 37°C and were then stained with EdU working mixture at 37°C for 24 h, followed by fixing with paraformaldehyde (Sangon Biotech Co., Ltd.) at room temperature for 10 min and permeabilization using Triton X-100 (Beyotime Institute of Biotechnology) at room temperature for 3 min. Subsequently, the cells were successively stained with an EdU reaction mixture for 30 min and Hoechst 33342 (both from Beyotime Institute of Biotechnology) for 10 min at room temperature, according to the manufacturer's protocol. An inverted fluorescence microscope (Motic Incorporation, Ltd.) was applied to capture images.

Cell apoptosis assay. NCI-H1650 and A549 cells were stimulated with 1 or 2 μ mol/l MI-2 for 24 h at 37°C and the cell apoptosis rate was determined using the Annexin V-IF488/PI Cell Apoptosis Detection Kit (Wuhan Servicebio Technology Co., Ltd.). Briefly, cells were washed and were then incubated with 5 μ l Annexin V and 5 μ l propidium iodide for 20 min at room temperature in the dark. Finally, cells were collected and cell apoptosis was assessed using the FACSCanto II flow cytometer (BD Biosciences) and analyzed by Flowjo X (FlowJo, LLC).

Cell migration and invasion assays. For the cell migration assay, NCI-H1650 and A549 cells were cultured to near confluence and a wound was created on the cell monolayer using a 10- μ l pipette tip. Cells were then washed to remove unattached cells prior to culturing for 24 h in serum-free medium with 1 or 2 μ mol/l MI-2. Images of the wound area were captured by an inverted light microscope (Motic Incorporation, Ltd.) and the cell migration rate was then calculated using the following formula: 1-wound area at 24 h/wound area at 0 h.

Additionally, cell invasion was assessed using a Transwell assay. Briefly, NCI-H1650 and A549 cells at a density of 3x10⁴ cells/well in serum-free medium supplemented with 1 or 2 μ mol/l MI-2 were seeded into 24-well plates pre-coated with Matrigel, at 37°C for 1 h, on the upper inserts (Corning, Inc.) of the Transwell chamber. The lower chamber was filled

with complete medium. Following incubation for 24 h, the invasive cells were stained with 0.1% crystal violet (Beyotime Institute of Biotechnology) at room temperature for 10 min and images were captured using an inverted light microscope (Motic Incorporation, Ltd.).

Western blot analysis. The BEAS-2B and NSCLC cell lines were cultured and the protein expression levels of MALT1 were determined by western blotting. In addition, NCI-H1650 and A549 cells were stimulated with 1 or 2 $\mu\text{mol/l}$ of MI-2 for 24 h at 37°C, and the protein expression levels of BCL2, BCL2-associated X-protein (BAX) phosphorylated (p)-c-JUN N-terminal kinase (JNK), JNK, p-c-JUN, c-JUN and GAPDH were detected by western blot analysis. Briefly, total proteins were extracted with a Cell Lysis Buffer (Cell Signaling Technology, Inc.) and were quantified using the Total Protein Assay Kit (Nanjing Jiancheng Bioengineering Institute). Subsequently, 20 μg proteins were separated by SDS-PAGE on 4-20% gels and were electrotransferred onto PVDF membranes. The membranes were then incubated with 5% non-fat powdered milk (Beyotime Institute of Biotechnology) at 37°C for 90 min and with primary antibodies (Affinity Biosciences) against the aforementioned proteins at an appropriate dilution, according to the supplier's instructions, at 4°C overnight. Following incubation with the corresponding secondary antibodies (Affinity Biosciences) at 37°C for 2 h, the protein bands were visualized using the Super ECL Detection Reagent (Shanghai Yeasen Biotechnology Co., Ltd.). The proteins were semi-quantified by ImageJ 1.8 (National Institutes of Health). The antibodies used were as follows: Anti-BCL2 (cat. no. AF6139; 1:2,000), anti-BAX (cat. no. AF0120; 1:2,000), anti-p-JNK (cat. no. AF3318; 1:2,000); anti-JNK (cat. no. AF6318; 1:1,500), anti-c-JUN (cat. no. AF6090; 1:2,000), anti-p-c-JUN (cat. no. AF3095; 1:2,000), anti-MALT1 (cat. no. DF6867; 1:3,000), anti-GAPDH (cat. no. AF7021; 1:3,000), and Goat Anti-Rabbit IgG (H+L) HRP (cat. no. S0001; 1:5,000) (all from Affinity Biosciences).

Anisomycin treatment assay. Anisomycin (MedChemExpress), an activator of the JNK pathway, was adopted to validate the modulation of the JNK pathway by MALT1. Briefly, NCI-H1650 and A549 cells were cultured and co-stimulated with 1 or 2 $\mu\text{mol/l}$ MI-2 and 0.1 $\mu\text{mol/l}$ anisomycin alone or in combination; the concentration of anisomycin (0.1 $\mu\text{mol/l}$) was determined based on a previous study (20). Following treatment for 24 h at 37°C, cells were collected for western blotting, EdU staining and cell apoptosis assays, which were conducted as aforementioned. Notably, the CCK-8 assay was carried out after 0, 24, 48 and 72 h of stimulation.

Statistical analysis. All analyses were performed using SPSS 22.0 software (IBM Corp.). Experiments were repeated in triplicate and data are presented as the mean \pm standard deviation. The differences among multiple groups were compared by one-way ANOVA followed by Dunnett's or Tukey's multiple comparisons test. The differences between two groups were compared by unpaired Student's t-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

MALT1 expression in NSCLC cell lines. The results showed that the mRNA expression levels of MALT1 were significantly increased in NCI-H1299 ($P < 0.05$), NCI-H1650 ($P < 0.001$), A549 ($P < 0.001$) and NCI-H23 ($P < 0.01$) cells compared with those in the BEAS-2B cell line. However, no significant difference was observed in the expression levels of MALT1 between HCC827 and BEAS-2B cells ($P > 0.05$; Fig. 1A). Consistent with the RT-qPCR analysis results, western blot analysis indicated that the protein expression levels of MALT1 were increased in NSCLC cells ($P < 0.05$; Fig. 1B). Notably, the mRNA and protein expression levels of MALT1 were highest in NCI-H1650 and A549 cells; therefore, these cell lines were chosen for subsequent experiments. Additionally, the CCK-8 assay demonstrated that the viability of MI-2-treated NCI-H1650 and A549 cells was decreased in a dose-dependent manner. The IC_{50} values of MI-2 in NCI-H1650 and A549 cells were 1.924 and 1.195 $\mu\text{mol/l}$, respectively (Fig. 1C).

Effect of MI-2 on NCI-H1650 and A549 cell proliferation, apoptosis, migration and invasion. The CCK-8 assay results revealed that the OD value in NCI-H1650 ($P < 0.001$) and A549 ($P < 0.01$) cells treated with MI-2 for 72 h was significantly decreased compared with that in the control group (Fig. 2A). In addition, the EdU staining assay showed that the EdU-positive rate was reduced in MI-2-treated NCI-H1650 ($P < 0.01$) and A549 ($P < 0.05$) cells compared with that in untreated cells (Fig. 2B). Furthermore, an Annexin V-IF488/PI Cell Apoptosis Detection Kit was used to determine cell apoptosis. As shown in Fig. 3A, the cell apoptosis rate was increased by MI-2 in NCI-H1650 ($P < 0.01$) and A549 ($P < 0.01$) cells. Furthermore, western blot analysis revealed that BAX was upregulated and BCL2 was downregulated in MI-2-treated NCI-H1650 and A549 cells compared with those in the control group (all $P < 0.05$; Fig. 3B). In addition, the cell migration assay demonstrated that MI-2 attenuated the migration of NCI-H1650 cells ($P < 0.05$), but not that of A549 cells ($P > 0.05$), compared with in untreated cells (Fig. 4A). Finally, the invasive ability of NCI-H1650 and A549 cells was assessed by Transwell assays. The results indicated that MI-2 reduced the invasion of NCI-H1650 cells ($P < 0.05$), but not that of A549 cells ($P > 0.05$), compared with in the control group (Fig. 4B).

Effect of MI-2 on the JNK/c-JUN pathway in NCI-H1650 and A549 cells. Western blot analysis was performed to detect the protein expression levels of p-JNK, JNK, p-c-JUN and c-JUN in NCI-H1650 and A549 cells. The results demonstrated that the p-JNK/JNK and p-c-JUN/c-JUN ratios were decreased in NCI-H1650 and A549 cells treated with MI-2 compared with those in the control group (all $P < 0.05$; Fig. 5).

Effect of anisomycin on the MI-2-mediated JNK/c-JUN pathway in NCI-H1650 and A549 cells. Furthermore, western blot analysis indicated that the p-JNK/JNK and p-c-JUN/c-JUN ratios were elevated in the anisomycin group compared with those in the control group (both $P < 0.001$). In addition, these protein ratios were enhanced in the MI-2 + anisomycin group vs. the MI-2 group in NCI-H1650 and A549 cells (all $P < 0.01$; Fig. 6).

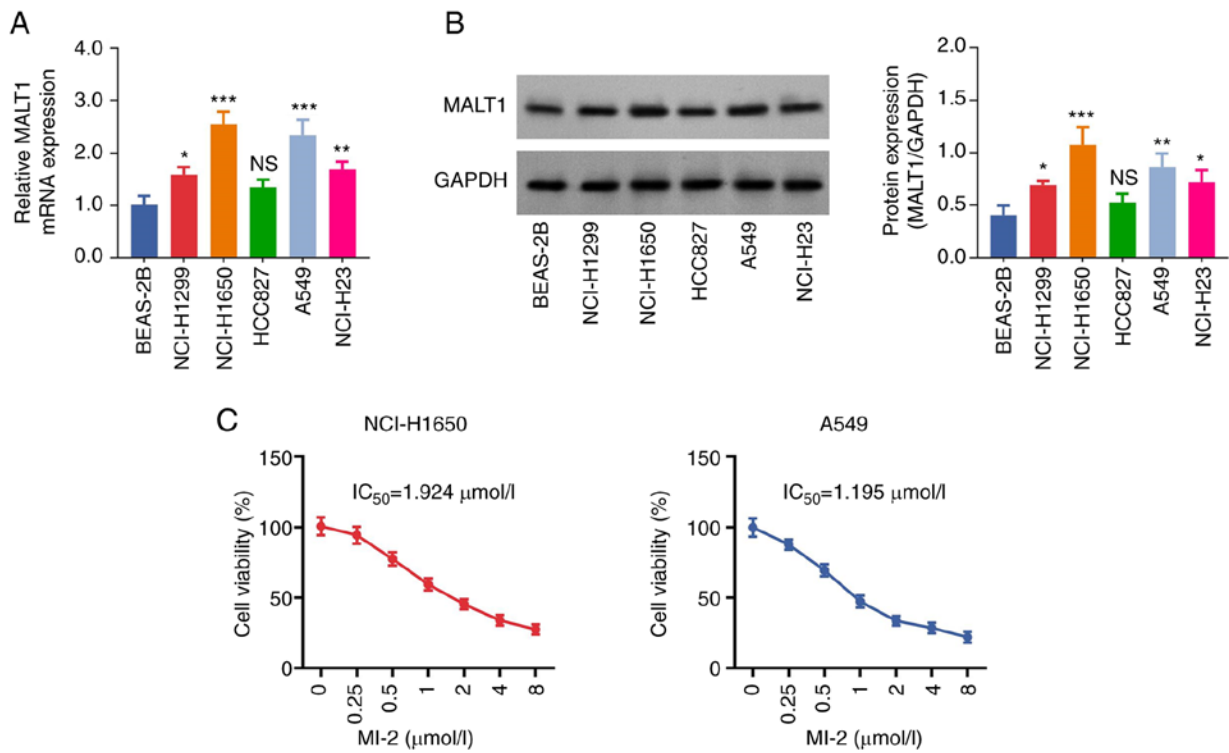


Figure 1. MALT1 is upregulated in non-small cell lung cancer cell lines. Comparisons in the (A) mRNA and (B) protein expression levels of MALT1 among the BEAS-2B, NCI-H1299, NCI-H1650, HCC827, A549 and NCI-H23 cell lines. (C) IC_{50} concentrations of MI-2 in NCI-H1650 and A549 cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. BEAS-2B. IC_{50} , half-maximal inhibitory concentration; MALT1, mucosa-associated lymphoid tissue lymphoma translocation protein 1.

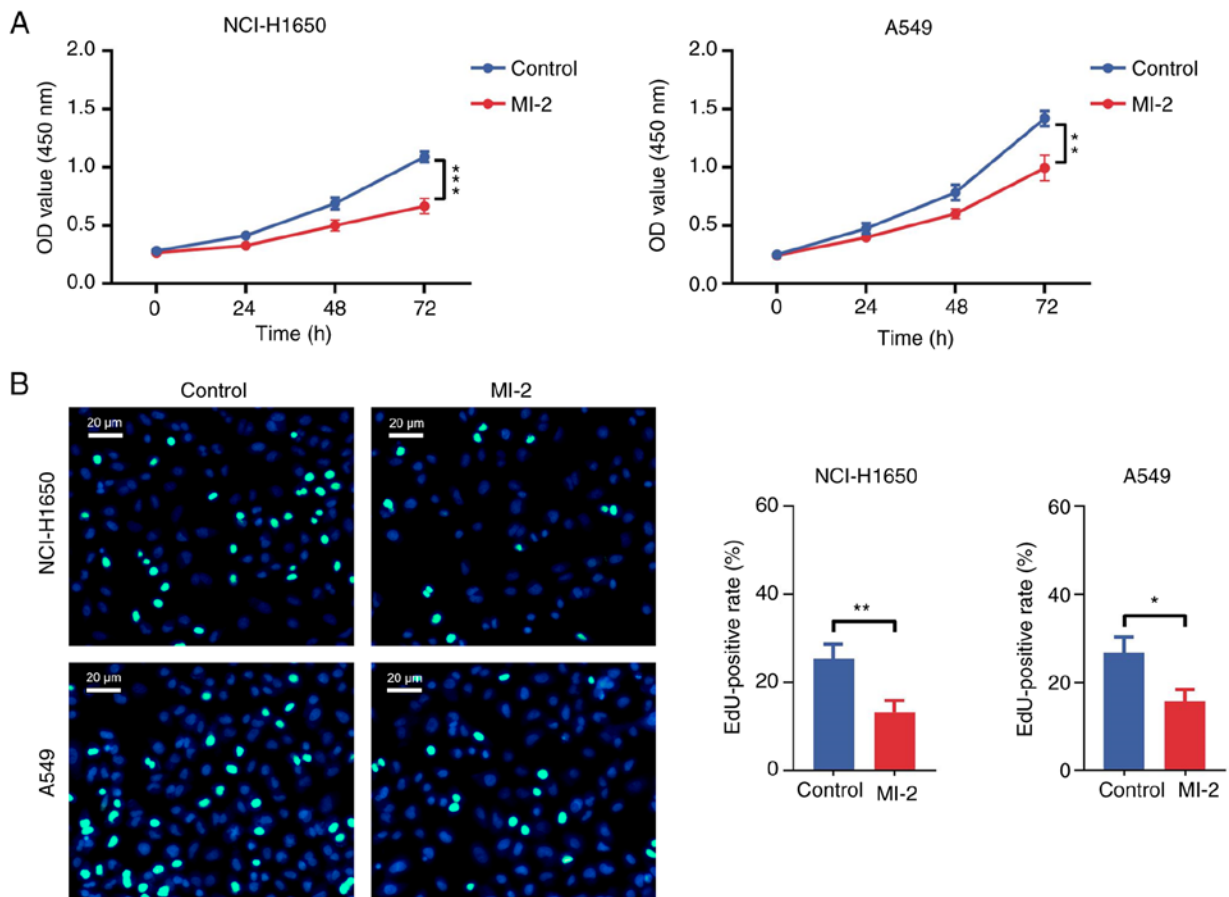


Figure 2. MI-2 inhibits the proliferation of NCI-H1650 and A549 cells. The effect of MI-2 on the proliferation of NCI-H1650 and A549 cells was assessed by (A) Cell Counting Kit-8 assay at different time points and (B) EdU staining assay. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. EdU, 5-ethynyl-2'-deoxyuridine; OD, optical density.

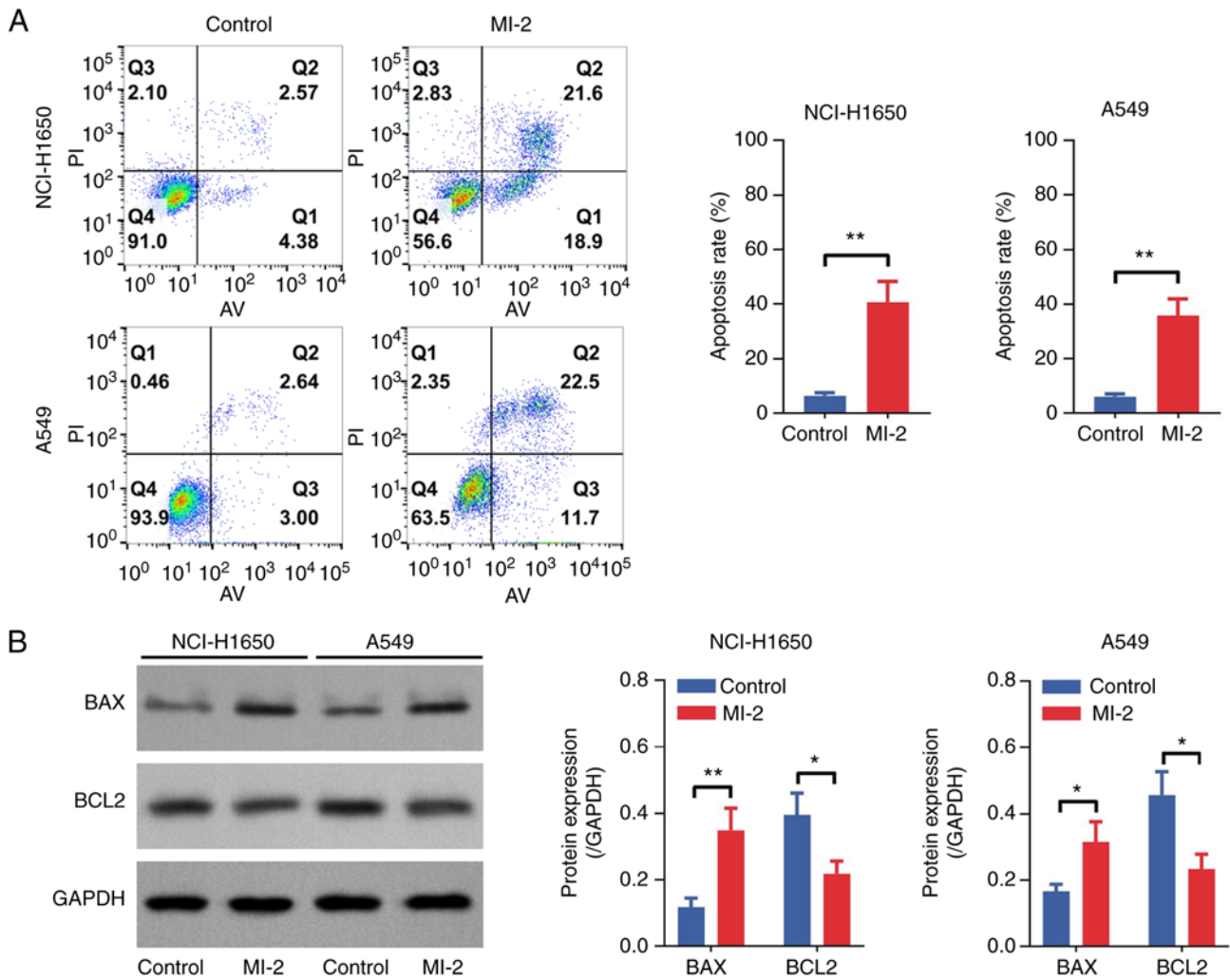


Figure 3. MI-2 promotes the apoptosis of NCI-H1650 and A549 cells. The effect of MI-2 on (A) NCI-H1650 and A549 cell apoptosis, and (B) expression levels of the apoptosis-related markers BAX and BCL2. *P<0.05, **P<0.01. BAX, BCL2-associated X-protein; BCL2, B-cell lymphoma 2.

Effect of anisomycin on MI-2-mediated NCI-H1650 and A549 cell proliferation and apoptosis. Subsequently, the CCK-8 assay revealed that the OD value was increased in the anisomycin group vs. the control group (both P<0.05), and in the MI-2 + anisomycin group vs. the MI-2 group (both P<0.01), in NCI-H1650 and A549 cells (Fig. 7A). The EdU staining assay demonstrated that the EdU-positive rate was not affected by anisomycin in NCI-H1650 cells (P>0.05), but it was increased in the anisomycin group compared with the control group in A549 cells (P<0.05). Consistently, the EdU-positive rate was elevated in the MI-2 + anisomycin group compared with that in the MI-2 group in both NCI-H1650 and A549 cells (all P<0.05; Fig. 7B). Additionally, the Annexin V-IF488/PI Cell Apoptosis Detection Kit showed that the cell apoptosis rate was not affected by anisomycin (both P>0.05), but it was reduced in the MI-2 + anisomycin group compared with that in the MI-2 group in both NCI-H1650 and A549 cells (both P<0.01; Fig. 8A). Western blot analysis indicated that BAX was only downregulated by anisomycin in A549 cells (P<0.05), whereas BCL2 was upregulated in both anisomycin-treated NCI-H1650 (P<0.01) and A549 (P<0.001) cells compared with in the control group. Furthermore, BAX was downregulated and BCL2 was upregulated in the MI-2 + anisomycin group

compared with in the MI-2 group in both NCI-H1650 and A549 cells (all P<0.05; Fig. 8B). These findings indicated that anisomycin may enhance proliferation but attenuate apoptosis in NCI-H1650 and A549 cells. Furthermore, anisomycin weakened the effect of MI-2 on inhibiting proliferation and facilitating apoptosis in NCI-H1650 and A549 cells.

Discussion

MALT1 is an indispensable unit of the CBM complex, which regulates malignant tumor cell survival, proliferation and metastasis, and is thus involved in the pathology and progression of some types of cancer (9). It has been reported that MALT1 is upregulated in several types of cancer (11,21). For example, a previous study elucidated that MALT1 is abundantly expressed in prostate cancer tissues compared with in normal tissues (22). Another study illustrated that MALT1 is elevated in colorectal cancer (CRC) tissues compared with in adjacent normal tissues (21). In the present study, the results showed that both the mRNA and protein expression levels of MALT1 were increased in NCI-H1299, NCI-H1650, A549 and NCI-H23 cells compared with in BEAS-2B cells. These finding could be due to the fact that MALT1 promotes cell

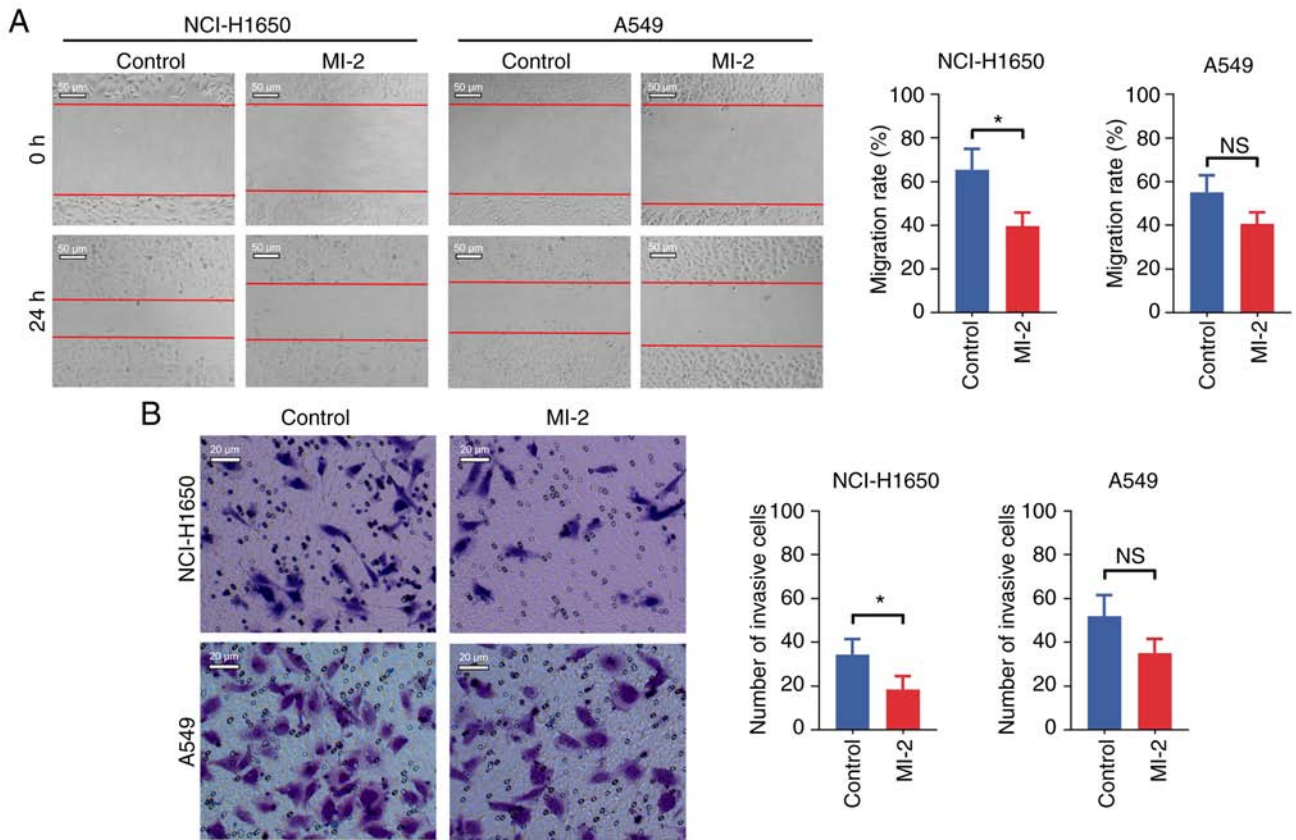


Figure 4. MI-2 inhibits the migration and invasion of NCI-H1650 cells. Effect of MI-2 on (A) migration and (B) invasion of NCI-H1650 and A549 cells. * $P < 0.05$. NS, not significant.

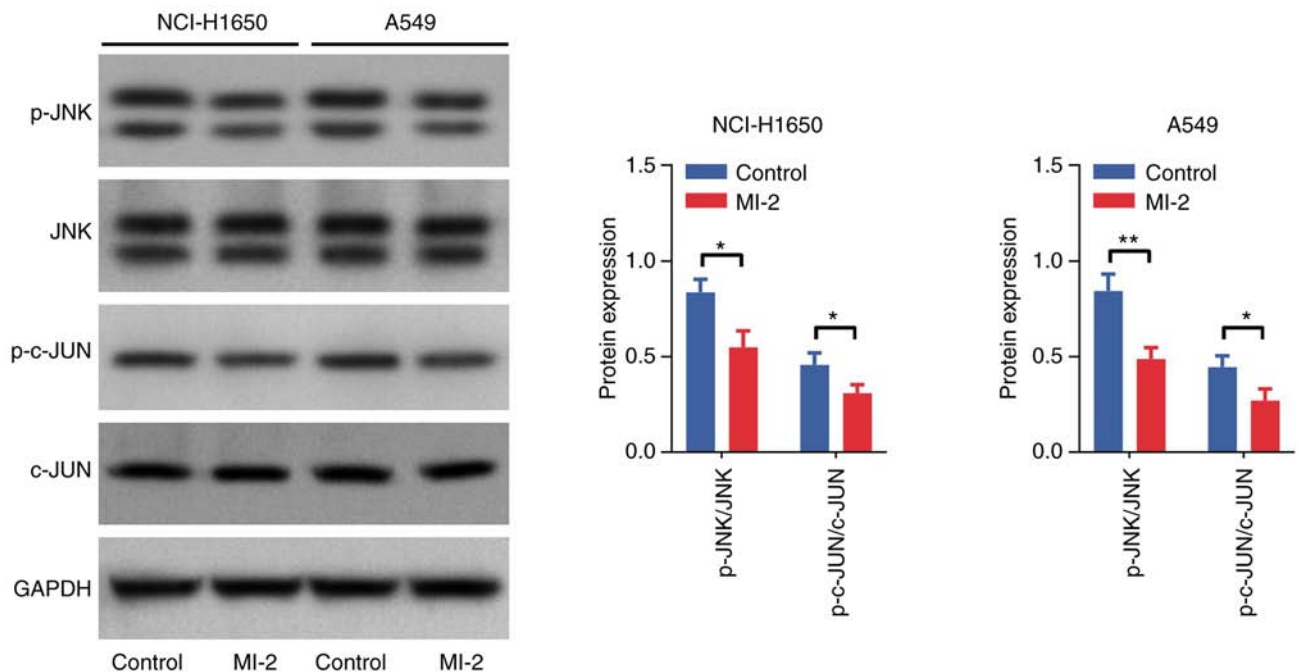


Figure 5. MI-2 suppresses the JNK/c-JUN pathway in NCI-H1650 and A549 cells. * $P < 0.05$, ** $P < 0.01$. JNK, c-JUN N-terminal kinase; p-, phosphorylated.

proliferation, thus suggesting that it could be involved in the malignant proliferation of NSCLC cells (11,23,24). Therefore, MALT1 was overexpressed in NSCLC cell lines compared with in normal lung epithelial cells. The identification of novel

biomarkers is fundamental for the early diagnosis of NSCLC, which assists in improving the prognosis of patients with NSCLC (25-27). The present findings provided a theoretical reference that MALT1 was highly expressed in NSCLC cells

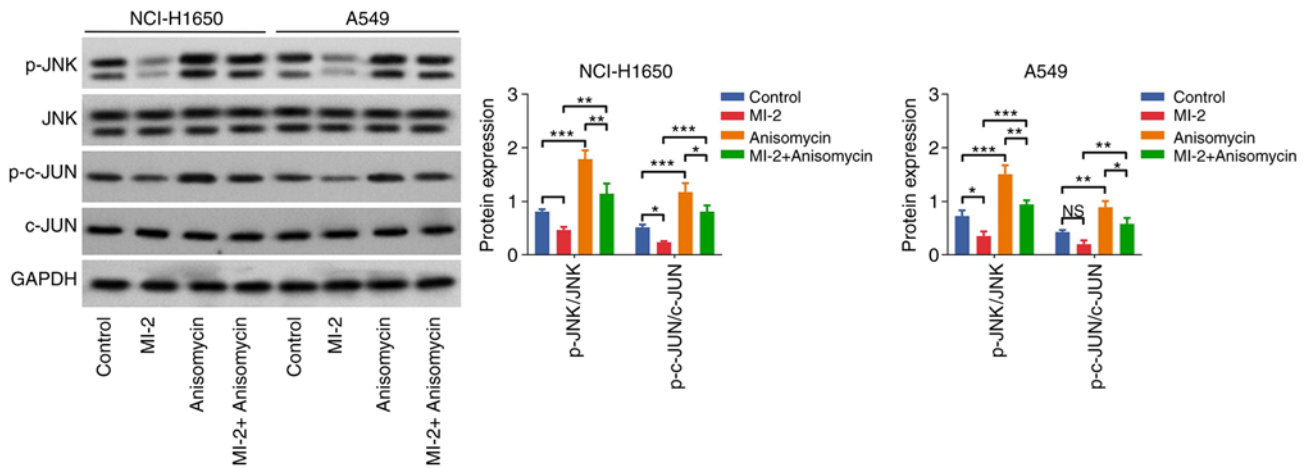


Figure 6. Anisomycin abrogates the effect of MI-2 on the JNK/c-JUN pathway in NCI-H1650 and A549 cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. JNK, c-JUN N-terminal kinase; NS, not significant; p-, phosphorylated.

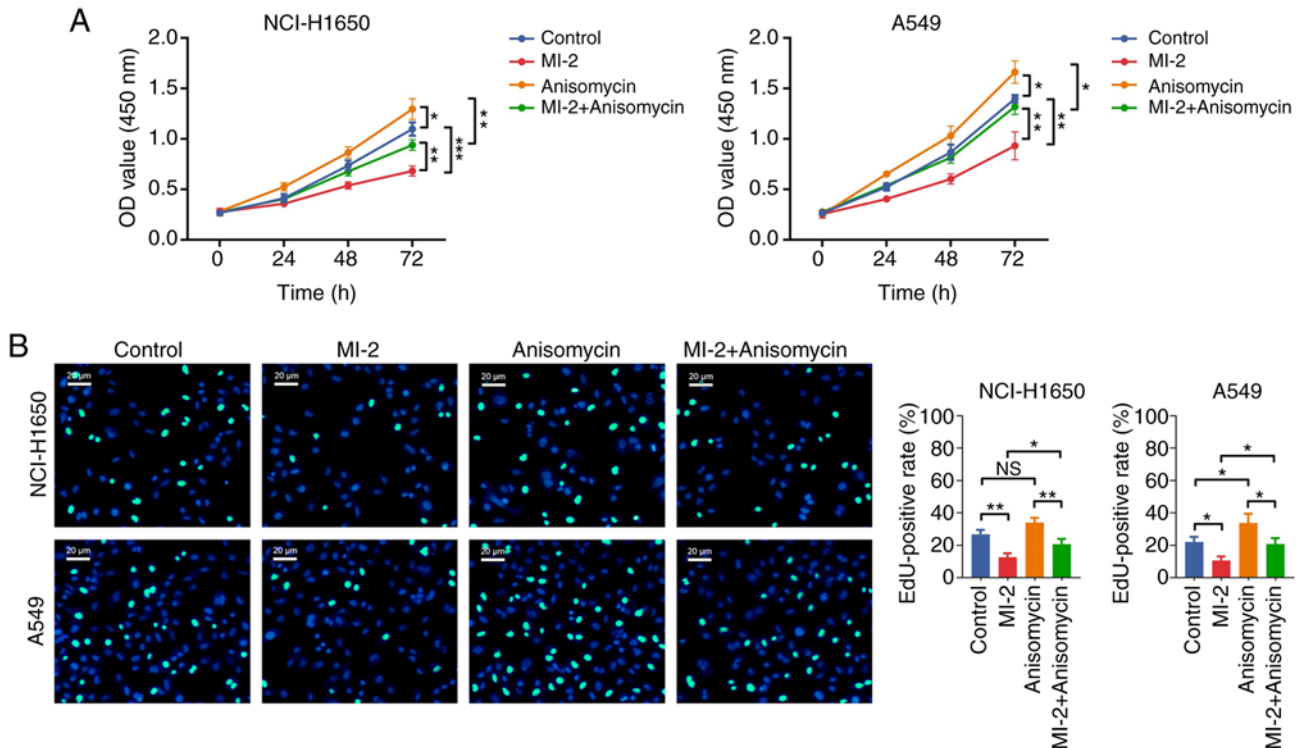


Figure 7. Anisomycin reverses the effect of MI-2 on the proliferation of NCI-H1650 and A549 cells. The effect of anisomycin on MI-2-mediated NCI-H1650 and A549 cell proliferation was determined by (A) Cell Counting Kit-8 and (B) EdU staining assays. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. EdU, 5-ethynyl-2'-deoxyuridine; OD, optical density.

compared with in normal cells, which suggested that MALT1 may possess the potential to serve as a biomarker for patients with NSCLC. However, this hypothesis should be validated by subsequent studies.

Targeting MALT1 is a potential strategy for attenuating cancer progression. Therefore, the role of MALT1 inhibitors, such as MI-2, on the progression of several types of cancer has been widely investigated (11,17,28,29). For example, a previous study reported that MI-2 can reduce A2058 and A375 melanoma cell proliferation and migration (29). Another study elucidated that MI-2 attenuates the migration, invasion and tumor-forming abilities of HCC cells, possibly via inhibiting

the NF- κ B pathway (11). Additionally, MI-2 can effectively inhibit glioblastoma multiforme cell proliferation, survival, migration and invasion (17), and can diminish the proliferation and migration of CRC cells (21). In the present study, the results suggested that MI-2 could reduce NSCLC cell proliferation, migration and invasion, and enhance cell apoptosis. These findings could be attributed to the possible effect of MI-2 on suppressing the activation of several signaling pathways, including the NF- κ B, B cell receptor, phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin and extracellular-signal-regulated kinase/mitogen-activated protein kinase pathways, to affect NSCLC cell proliferation,

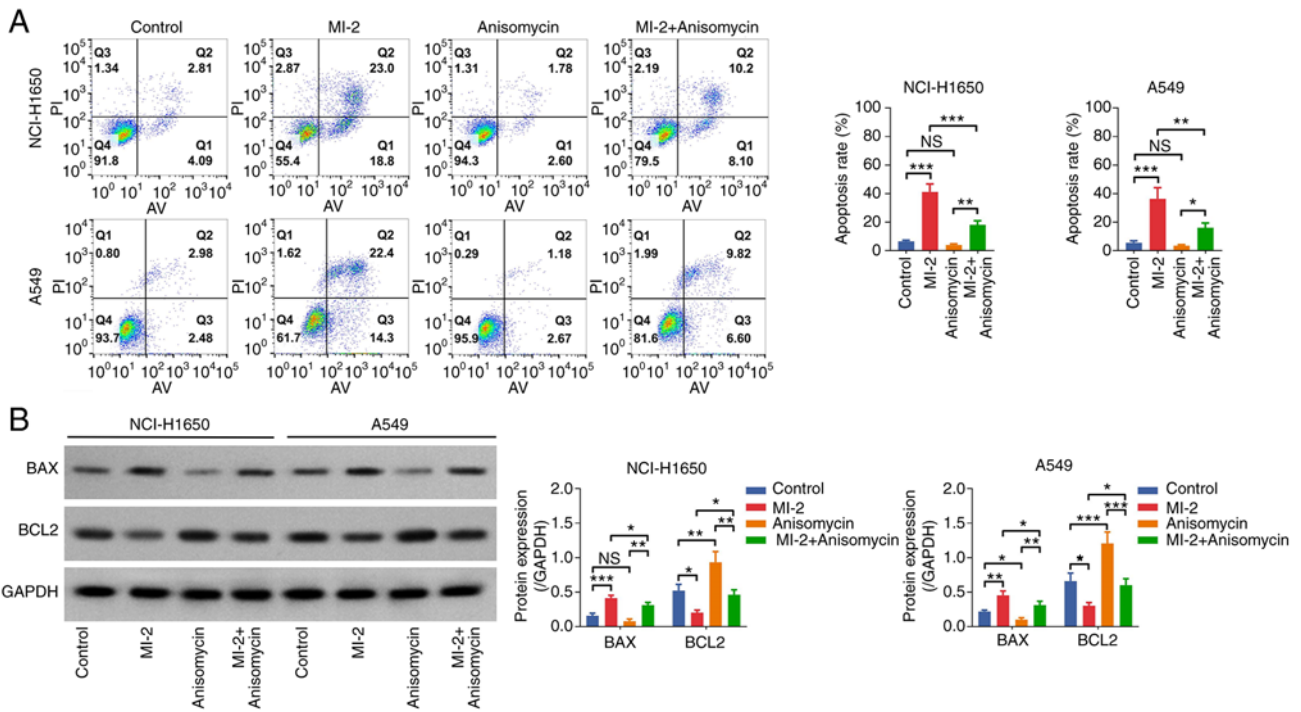


Figure 8. Anisomycin attenuates the effect of MI-2 on the apoptosis of NCI-H1650 and A549 cells. Effect of anisomycin on MI-2-mediated (A) cell apoptosis and (B) expression levels of the apoptosis-related markers, BAX and BCL2, in NCI-H1650 and A549 cells. *P<0.05, **P<0.01, ***P<0.001. BAX, BCL2-associated X-protein; BCL2, B-cell lymphoma 2.

apoptosis, migration and invasion (18,24,30,31). Notably, MI-2 could only reduce migration and invasion in NCI-H1650 cells, but not in A549 cells; the reasons for this may be: i) According to a previous study, metastatic cell lines with high MALT1 expression are particularly sensitive to MI-2 (29). In addition, according to the American Type Culture Collection, NCI-H1650 cells were derived from the metastatic sites of the lung, whereas A549 cells were not. Therefore, the inhibitory effect of MALT1 by MI-2 might have a limited effect on reducing A549 cell invasion and migration compared with NCI-H1650 cells. However, further experiments are required to validate this speculation. ii) Since the number of experimental repeats was only three, the statistical power would be affected. To further confirm the findings, more experimental repeats are recommended, preferably at least five. Moreover, it should be clarified that MI-2 is a potent MALT1 inhibitor, which acts by directly binding to MALT1 and inhibiting its protease function (16). Thus, it was hypothesized that MI-2 could only inhibit the protease function of MALT1, but it could not affect its expression, which was confirmed by a previous study (32). However, this speculation should be validated by subsequent experiments.

Emerging evidence has suggested that the JNK/c-JUN pathway is closely involved in the pathology and progression of NSCLC (33-36). For example, a previous study showed that the activated JNK/c-JUN pathway promotes the growth and metastasis of lung adenocarcinoma (33). Additionally, inhibition of the JNK/c-JUN pathway accelerates NSCLC cell apoptosis (36). Furthermore, another study found that activation of the JNK/c-JUN pathway could induce NSCLC cell proliferation and migration, and attenuate NSCLC cell apoptosis (35). Regarding the regulatory effect of MALT1 on the

JNK/c-JUN pathway, a previous study indicated that MALT1 activates the JNK/c-JUN pathway to facilitate melanoma cell proliferation and motility (29). However, whether the regulatory effect of MALT1 on the JNK/c-JUN pathway also participates in the progression of NSCLC remains unclear. In the current study, the results demonstrated that MI-2 suppressed the JNK/c-JUN pathway in NSCLC cells. This could be due to the fact that MI-2 can degrade the CBM complex or regulate cleavage of the deubiquitinase cylindromatosis (CYLD), thus further inactivating JNK/c-JUN signaling (23,37). In addition, the present study showed that co-treatment of NSCLC cell with anisomycin and MI-1 abrogated the effects of MI-2 on the JNK/c-JUN signaling pathway, along with its effect on NSCLC cell proliferation and apoptosis. As aforementioned, it was hypothesized that MI-2 could affect the CBM complex or CYLD cleavage, thus inactivating the JNK/c-JUN signaling pathway, ultimately attenuating NSCLC cell proliferation and promoting NSCLC cell apoptosis (23,37). However, whether the regulatory effect of MI-2 on JNK/c-JUN signaling is direct or indirect should be verified by further experiments.

In conclusion, the present study demonstrated that MI-2, a MALT1 inhibitor, could impair NSCLC cell proliferation, migration and invasion, and promote NSCLC cell apoptosis by suppressing the JNK/c-JUN pathway, which could be involved in attenuating NSCLC progression. To the best of our knowledge, this is the first study that explores the involvement of MI-2 in NSCLC, and our findings suggested that MI-2 may be helpful in attenuating NSCLC progression. Based on the findings of this study, further studies could consider exploring the effect of MI-2 on other types of cancer. Meanwhile, other potential pathways involved in the regulation of MI-2 on NSCLC could be further investigated.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

CW, WG and YW contributed to the study design, collected and analyzed data, and contributed to the paper writing and reviewing. CW and WG performed the experiments. WG and YW confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethical approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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