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Short communication

Curcumin inhibits migration and invasion of non-small cell lung cancer cells through up-regulation of miR-206 and suppression of PI3K/AKT/mTOR signaling pathway

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Curcumin has been proved to inhibit cell proliferation and induce cell apoptosis in non-small cell lung cancer (NSCLC). However, little is known about antimetastatic effects and molecular mechanisms of curcumin in NSCLC. In this study, we investigated the involvement of miR-206 in curcumin's anti-invasion and anti-migration in NSCLC. Cell proliferation was determined by MTT assay. Cell migration and invasion were analyzed by wound healing assay and transwell assay. MiRNA-206 expression was detected by real-time PCR. Western blot was used to detect the protein expression of PI3K/AKT/mTOR signaling pathway. Curcumin significantly inhibited migration and invasion in A549 cells, accompanied by significantly elevated miR-206 expression. Overexpression of miR-206 could inhibit migration and invasion of A549 cells, but it could also significantly decrease the phosphorylation levels of mTOR and AKT. The inhibition of miR-206 promoted cell migration, invasion and increased the phosphorylation level of mTOR and AKT. Furthermore, miR-206 mimics improved the inhibitory effects of curcumin on cell migration, invasion and the phosphorylation level of mTOR and AKT in A549 cells. On the contrary, MiR-206 inhibitors reversed the inhibitory effects of curcumin on cell migration, invasion and the phosphorylation level of mTOR and AKT. In conclusion, curcumin inhibited cell invasion and migration in NSCLC by elevating the expression of miR-206 which further suppressed the activation of the PI3K/AKT/mTOR pathway.

Keywords: curcumin, NSCLC, microRNAs, migration and invasion

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Lung cancer is a common malignant tumor worldwide with approximately 80 to 85 % of all lung cancers being accounted for non-small cell lung cancer (NSCLC). The incidence of lung cancer nearly doubled in China over the past ten years. To date, advanced achieve-

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ment has been made in the treatment of NSCLC. However, many patients with NSCLC are presented with metastasis to existing treatments, such as chemotherapy, radiotherapy and surgery (1).

MicroRNAs are small non-codding RNA molecules that regulate a variety of cellular processes by binding to the 3'-untranslated region of their target genes (2). In the past decade, accumulated research has indicated that miRNAs play a key regulatory role in the occurrence and development of diverse types of cancer, including lung cancer. These studies suggested that miRNAs could be novel diagnostic and therapeutic markers. For instance, overexpression of miR-485-5p has been suggested to reduce cell migration and invasion *via* targeting flotillin-2 in small cell lung cancer (3). MiR-1236-3p had been linked to DDP resistance in NSCLC (4). In addition, miR-206 plays a tumor-suppressing role in lung cancer by suppressing the NSCLC growth, cisplatin resistance, and metastasis *via* targeting Bcl2, c-met, Sox9 and cyclinD1 (5).

Curcumin, a natural polyphenol obtained from the traditional medicine *Curcuma longa* L. (Zingiberaceae), has been investigated widely for its effects for thousands of years (6). Several studies have reported that curcumin not only has anti-inflammatory and anti-oxidant, but also anti-tumor effects (7). The anticancer effect of curcumin was considered to inhibit cell proliferation by suppressing multiple cell signaling pathways (8). For example, curcumin could induce apoptosis by regulating the miR-21/PTEN/AKT signaling pathway in MGC-803 cells (9). Furthermore, curcumin inhibited cell migration and invasion by regulating miR-99a and JAK/STAT signaling pathway in retinoblastoma cells (10). Although the anti-tumor effects of curcumin have been studied, the exact mechanisms by which curcumin affects the NSCLC are still poorly understood.

Since curcumin could change the expression profile of miRNA in various types of cancer (11), we hypothesize that curcumin may suppress cell migration and invasion of NSCLC through miR-206. Thus, the effects of curcumin and miR-206 on invasion and migration capabilities of A549 cells were investigated. Furthermore, the regulation effect of curcumin on the expression of miR-206, which then inhibited migration and invasion in A549 cells via attenuating the PI3K/AKT/mTOR signaling pathway was also examined to demonstrate that curcumin suppresses invasion and metastasis of NSCLC by increasing miR-206 expression.

EXPERIMENTAL

Cell culture and reagents

The A549 cells were obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultivated in RPMI-1640 media supplemented with 10 % fetal bovine serum (Gibco, Austria) in a humidified incubator at 37 °C in an atmosphere of 5 % CO_2. The medium contained 100 U mL^{-1} penicillin and 100 g mL^{-1} streptomycin. Curcumin was purchased from Sigma (China). The purity of curcumin was analyzed by HPLC and is over 99.5 %. Mimic negative control (mimic NC), inhibitor NC, miR-206 mimics and miR-206 inhibitors were purchased from GenePharma (China). Primary antibodies AKT, phospho-AKT(S473), mTOR and phospho-mTOR(S2448) were purchased from Cell Signaling Technology (USA).

Cell viability assays

Cell viability was detected by MTT assay. The A549 cells were inoculated in 96-well plates (5×10^3 cells/well) and maintained in RPMI-1640 media supplemented with 10 % FBS. The A549 cells were cultured overnight, the medium was removed and replaced with a new medium. Then, 0, 5, 10, 20 and 40 μmol L^{-1} curcumin was added to 96-well plates and cultured for another 24 hours at 37 °C in an atmosphere of 5 % CO_2. Four hours before the experiment, A549 cells were incubated in the MTT solution at a density of 0.5 mg mL^{-1}. Lastly, the medium with MTT solution was removed and 150 μL of DMSO was added to each well. A549 cells were subjected to absorbance measuring by the use of a Microplate reader at 490 nm. Each experiment was independently performed 3 times.

MicroRNA transfection

MiR-206 mimics, mimic NC, miR-206 inhibitors and inhibitor NC transfected into A549 cells by using Lipofectamine® 2000 reagent (Invitrogen, Carlsbad, CA, USA). Firstly, the A549 cells were plated in 6-well plates (1×10^5 cells/well) for 24 hours. Secondly, when the density of cells reached 70 %, the A549 cells were transfected with miR-206 mimics, mimic NC, miR-206 inhibitors or inhibitor NC for 6 hours according to the instructions of Lipofectamine 2000. Finally, the transfected A549 cells were cultivated in new medium containing 10 % FBS for another 24 hours.

Migration and invasion assay

The cell migration and invasion were performed by wound healing assay and transwell assay. For wound healing assay, the A549 cells were cultured in 6-well plates overnight. The wound was generated by scratching the cell layer using a 10 μL pipette tip when A549 cells reached approximately 90 % confluency. In order to remove non-adherent cells, the well plates were washed with PBS and fresh medium without FBS was added for 24 hours. Then the wound healing effect was detected under microscope at 0 and 24 h. ImageJ software measured the area of each wound. Transwell assay was performed in 24-well chambers coated with matrigel. A549 cells (3×10^4 cells/well) were plated in 200 μL medium without FBS and added into the upper chamber. Subsequently, 600 μL medium with 10 % FBS was added to the lower chamber. After the incubation for 24 hours, A549 cells that have invaded into the lower surface were fixed with ice methanol and stained with crystal violet. Finally, the invaded cells were photographed under a microscope at the magnification of 200×.

Real-time PCR analysis

The design of RT-PCR primers was based on miR-206 sequences which were provided by the sanger center miRNA registry. Total RNA was extracted from A549 cells which were transfected with miR-206 mimics, mimic NC, miR-206 inhibitors or inhibitor NC by using TRIzol reagent (Invitrogen). Then, according to the manufacturer's protocol, 2 μg of total RNA has synthesized complementary DNA by AMV reverse transcriptase (Promega, Madison, WI, USA). The expression level of miR-206 was detected by mirVana™ qRT-PCR

miRNA Detection kit (Invitrogen). Meanwhile, the expression level of U6 was used as internal control. We used $2^{-\Delta\Delta CT}$ method to calculate the expression levels of target gene.

Western blotting

Total proteins were extracted from A549 cells using radioimmunoprecipitation assay (RIPA) lysis buffer containing PMSF and protease inhibitors. The protein concentration in each group was determined by the BCA protein assay. Equivalent protein was separated on 10 % SDS-PAGE at 80 V for 2 hours. Then the separated proteins were transferred to PVDF membranes at 350 mA for 2 hours. The membranes were treated with 5 % BSA at room temperature for one hour to block nonspecific binding, followed closely by the incubation with primary antibodies at 4 °C overnight. The used primary antibodies were rabbit anti-phospho-AKT (1:1000 dilution), rabbit anti-phospho-mTOR (1:1000 dilution), rabbit anti-AKT antibody (1:1000 dilutions) and rabbit anti-mTOR antibody (1:1000 dilution). The membranes were washed with TBST to remove unbounded antibodies. Secondary antibodies (HRP-labeled goat anti-rabbit IgM antibody) were added and the membranes were incubated for 1h at room temperature. Protein signals were revealed using ECL, and the intensities of scanned bands were performed using the ImageJ software.

Statistical analysis

Statistical analyses of the experimental results were performed using the SPSS 17.0. All data were expressed as mean ± SD values. Data were graphed by GraphPad Prism 5.0 and analyzed by using one-way analysis of variance with the Tukey multiple comparison test. All experiments were performed at least three times. $p < 0.01$ could be considered as statistically significant.

RESULTS AND DISCUSSION

Curcumin inhibits cell proliferation in A549 cells

Curcumin, a famous traditional Chinese medicine, has chemo-preventive and therapeutic properties against a variety of tumors. Studies have shown that curcumin could suppress tumorigenesis, inhibit tumor promotion, and suppress angiogenesis (12). Curcumin could not only inhibit cell proliferation of tumor cells, but could also inhibit the proliferation of normal cells (13). For example, curcumin significantly inhibited the proliferation of BEAS-2B cells at the concentration of 10 μmol L^{-1} (14). To confirm whether curcumin suppresses cell proliferation, A549 cells were treated with curcumin at different concentrations for 24 h, and cell viability was detected by MTT assay. The results indicated that curcumin had significantly inhibited cell proliferation at concentrations of 10, 20 and 40 μmol L^{-1}, compared with the control group, with average rates of cell viabilities of 79.77 ± 2.49 %, 68.95 ± 3.63 % and 55.53 ± 2.84 %, respectively (Fig. 1). These data showed that curcumin significantly inhibited the proliferation of A549 cells in a dose-dependent manner.

Curcumin inhibits migration and invasion in A549 cells

Recently, it was reported that the antitumor effect of curcumin had mainly focused on anti-invasion and anti-metastasis (15). However, the effects of curcumin on migration,

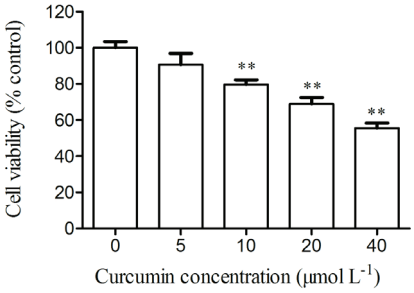

Fig. 1. The effects of curcumin on the viability of A549 cells. A549 cells were treated with curcumin in a gradient concentration (0–40 μmol L^{-1}) at 24 h. Data are presented as the mean ± SD, $n = 3$; $^{**}p < 0.01$ *vs.* control.

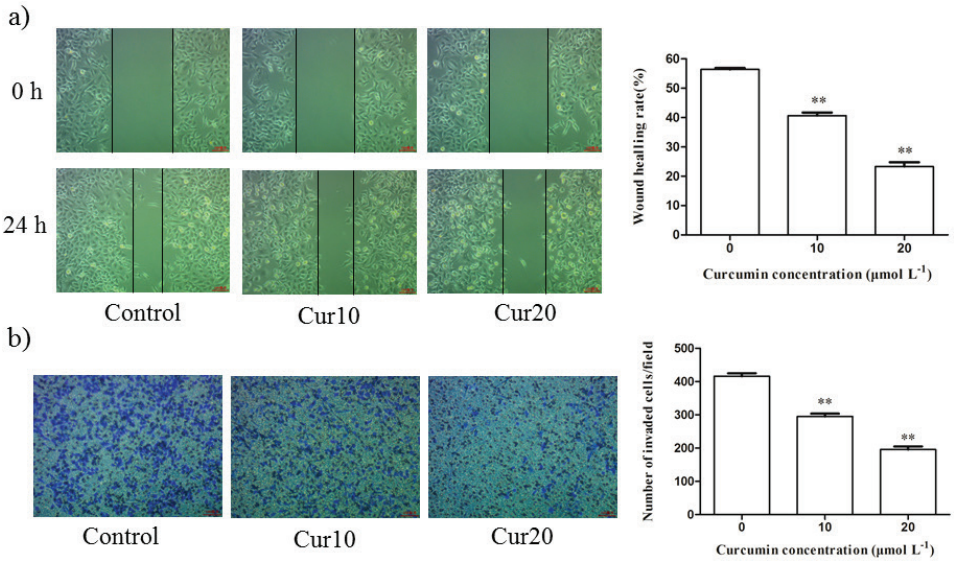

Fig. 2. Curcumin inhibited cell migration and invasion in A549 cells. a) A549 cells were scratched with a pipette tip and then treated with 10 and 20 μmol L^{-1} curcumin. Representative images showing the inhibitory effect of curcumin on cell migration at 24 h; b) representative images of cells that migrated through the polycarbonate membranes in transwell assays stained with crystal violet. Columns, mean from three different experiments with three duplicates, $^{**}p < 0.01$ *vs.* control.

invasion and potential molecular mechanisms in lung cancer remain largely unclear. After identifying the role of curcumin in cell proliferation, we further investigated its effects on migration and invasion of A549 cells according to the literature (6). As shown in Fig. 2, the A549 cells were treated with curcumin (0, 10 and 20 μmol L^{-1}) closed 56.42 %, 40.70 % and 23.35 % of the wounded area for 24 h, respectively. Consistent with the results of wound

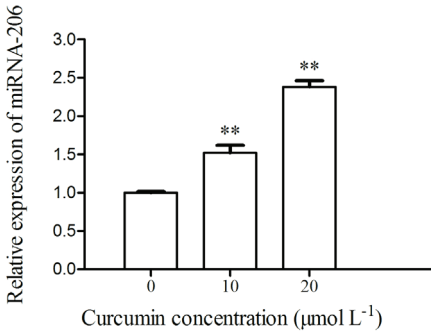

Fig. 3. Relative expression of miR-206 in A549 cells treated with curcumin was detected by real-time PCR. A549 cells were treated with different concentrations of curcumin for 24 h and miRNA fold-inductions by curcumin were evaluated by real-time PCR. $^{**}p < 0.01$ *vs.* control.

healing, the number of invaded cells has significantly decreased compared to untreated cells. The invasion capacity of A549 cells decreased 29.01 % and 52.96 %, respectively, compared with the control group at the concentration of 10 and 20 μmol L^{-1} of curcumin (Fig. 2). Taken together, the above results indicate that curcumin could suppress cell migration and invasion in NSCLC.

miR-206 is upregulated in curcumin-treated A549 cells

MiRNA dysregulation widely occurred in NSCLC, which might contribute to tumor occurrence and progression (16). To verify whether curcumin could regulate the expression of miR-206 in A549 cells, we treated the A549 cells with 10 and 20 μmol L^{-1} curcumin for 24 h. Then the relative expression of miR-206 was detected by real-time RT-PCR. As shown in Fig. 3, curcumin has significantly increased the expression level of miR-206 compared to the control group in A549 cells. When the curcumin concentration had been increased to 20 μmol L^{-1}, the expression level of miR-206 increased 2.37-fold relative to the untreated cells. It has been reported that miR-206 mimics could suppress migration and invasion in NSCLC by targeting c-met (17). Therefore, miR-206 could regard as a potent tumor suppressor. In this study, we have found that the treatment of curcumin had elevated the expression of miR-206 in A549 cells, suggesting that the regulation of miR-206 expression may be correlated with curcumin's anti-migration and anti-invasion.

miR-206 was involved in the effects of curcumin on cell migration and invasion in A549 cells

In order to investigate how miR-206 affected the effects of curcumin on migration and invasion of A549 cells, we conducted microRNA transfection. We transfected miR-206 mimics, mimic NC, miR-206 inhibitors and inhibitor NC into A549 cells to change the expression of miR-206. As shown in Fig. 4a, the mimics and inhibitors of miRNA-206 significantly upregulated and downregulated the miRNA-206 expression in A549 cells, respectively. As shown in Fig. 4b and 4c, miR-206 mimics significantly suppressed migration and invasion in A549

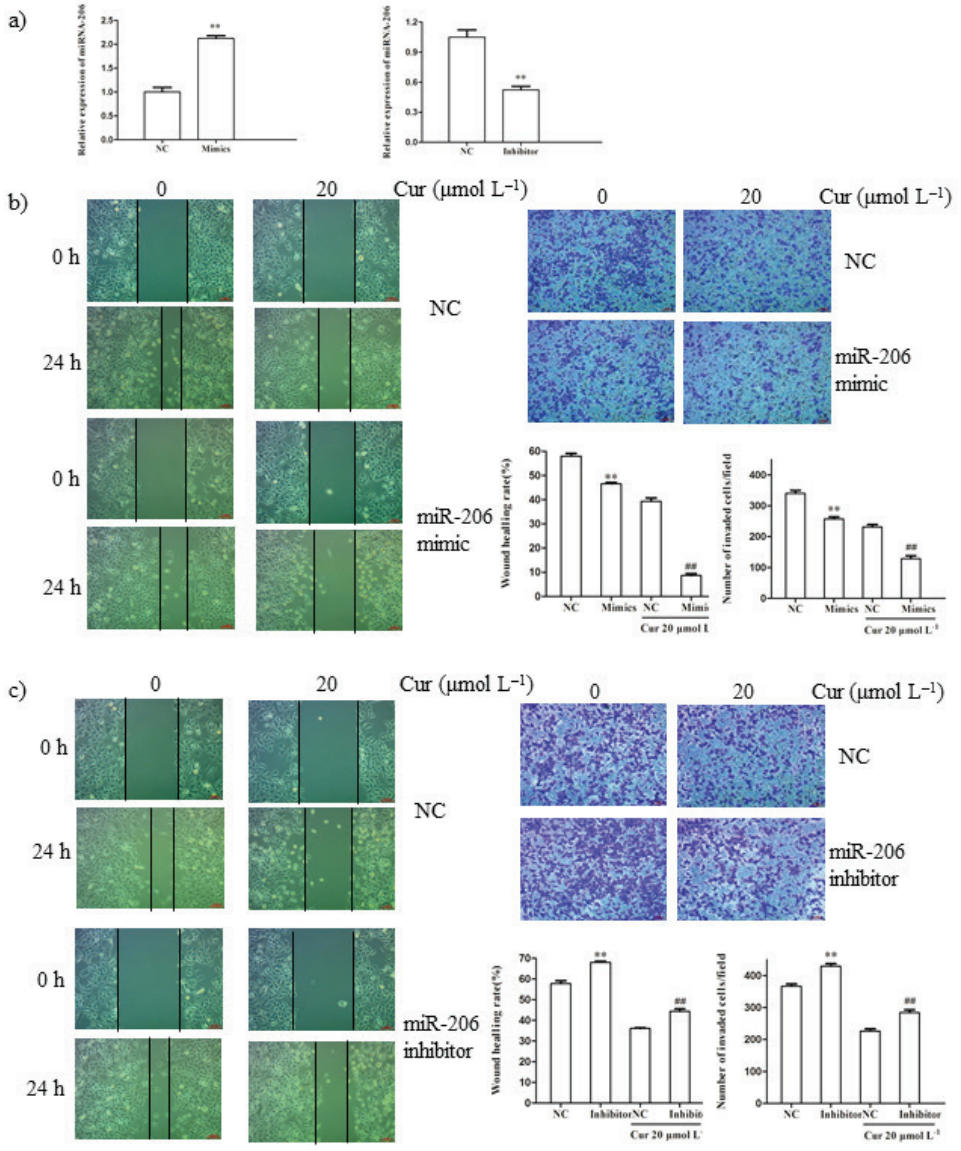

Fig. 4. miR-206 inhibits the effect of curcumin on A549 cell migration and invasion. a) A549 cells were transfected with miR-206 mimics and miR-206 inhibitors for 24 h. Real-time PCR was used to evaluate the expression level of miR-206; b) and c) A549 cells were transfected miR-206 mimics or miR-206 inhibitors, and then treated with 20 μmol L^{-1} curcumin, wound-healing assays and transwell invasion assays were used to investigate the migratory and invasive ability of A549 cells, respectively. Columns, mean from three different experiments with three duplicates, $^{**}p < 0.01$ compared with negative control and $^{\#\#}p < 0.01$ compared with curcumin + negative control.

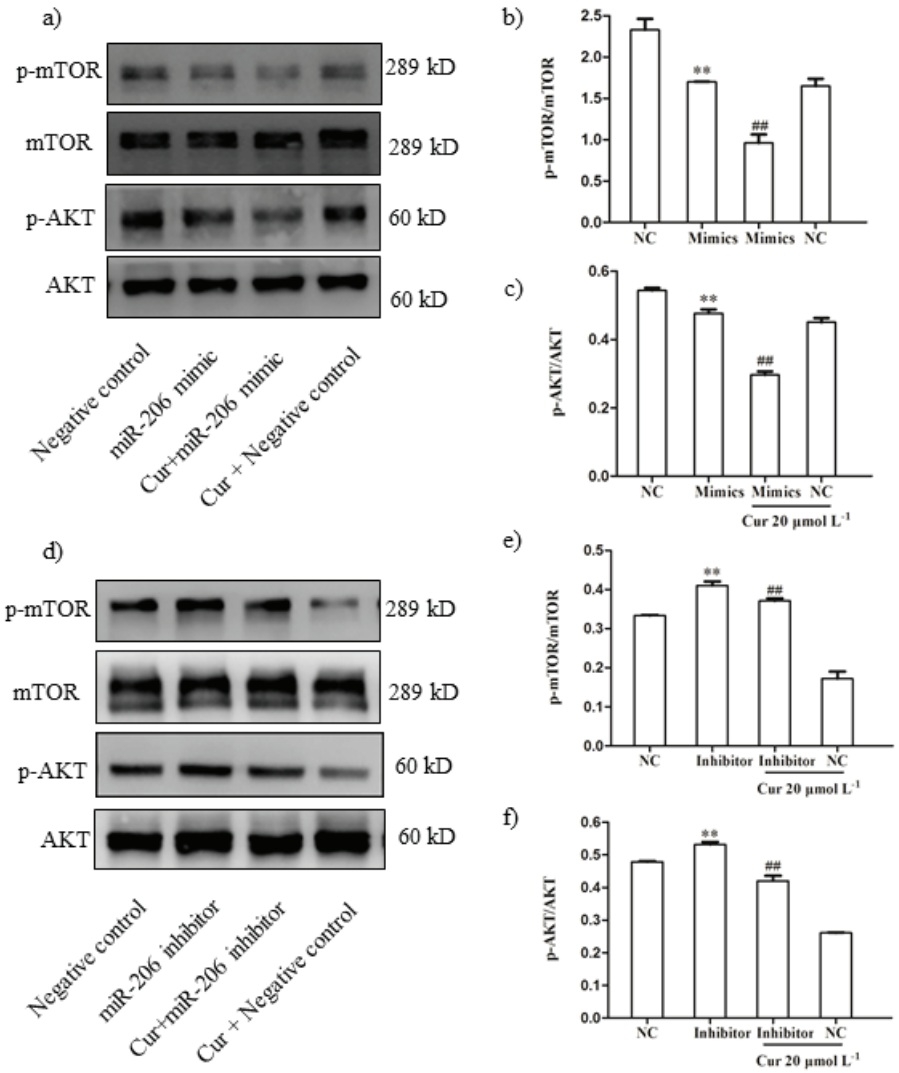

Fig. 5. Curcumin increased miR-206 expression to partly prevent the PI3K/AKT/mTOR signaling pathway. a) and d) Western blot analysis of the expression levels of p-mTOR, mTOR, p-AKT and AKT in A549 cells; b), c), e) and f) quantitative data of the levels of p-mTOR and p-AKT. Data are presented as the mean ± SD, $n = 3$; $^{**}p < 0.01$ compared with negative control and $^{\#\#}p < 0.01$ compared with curcumin + negative control.

cells, while miR-206 inhibitors markedly promoted migration and invasion in A549 cells. Moreover, miR-206 mimics transfection markedly increased inhibition of curcumin on migration and invasion. On the contrary, the miR-206 inhibitor's transfection had remarkably reversed the curcumin's inhibition of migration and invasion in A549 cells.

Curcumin increases miR-206 expression to partly prevent PI3K/AKT/mTOR signaling pathway

The PI3K/AKT/mTOR signaling pathway is commonly activated in NSCLC. Moreover activation of this pathway could promote tumor development through gene mutations, PI3K mutation or amplification, oncogene receptor activation and AKT mutation or amplification (18). Accumulating studies have reported that the PI3K/AKT/mTOR signaling pathway is one of the downstream targets of miR-206 (19, 20), therefore, we hypothesized that curcumin inhibited migration and invasion in A549 cells through suppressing PI3K/AKT/mTOR signaling pathway. Then, we transfected miR-206 mimics, mimic NC, miR-206 inhibitors and inhibitor NC into A549 cells for 24 h. As shown in Fig. 5, miR-206 mimics have decreased the phosphorylation level of mTOR and AKT, whereas no significant changes were observed in the total mTOR and AKT levels. However, miR-206 inhibitors have activated the phosphorylation level of mTOR and AKT. Furthermore, the combined treatment with curcumin and miR-206 mimics could remarkably decrease the phosphorylation levels of mTOR and AKT. On the contrary, the combined treatment with curcumin and miR-206 inhibitors has partially prevented curcumin's reduction of the phosphorylation levels of AKT and mTOR. These data have demonstrated that curcumin might up-regulate miR-206 expression to partly alleviate PI3K/AKT/mTOR signaling pathway.

CONCLUSIONS

In this study, we have found the evidence supporting the mechanisms of curcumin's anti-invasion and anti-metastatic effects were correlated with miR-206 in A549 cells. The abilities of invasion and migration were significantly inhibited by curcumin in A549 cells for 24 h, during which the expression level of miR-206 was determined. The results indicated that the expression level of miR-206 was markedly higher than that in control group. Furthermore, the combined treatment with curcumin and miR-206 mimics could remarkably decrease the phosphorylation levels of mTOR and AKT. On the contrary, combining curcumin treatment with miR-206 inhibitors partially prevented curcumin suppressing the phosphorylation level of mTOR and AKT. In summary, this study was the first one revealing the involvement of miR-206 in curcumin's anti-invasion and anti-migration effects in NSCLC. Based on the results, we concluded that curcumin inhibited A549 cell migration and invasion *via* elevating the expression of miR-206 and suppressing activation of the PI3K/AKT/mTOR pathway. The above results of the present study reveal that curcumin may be a potential therapeutic drug for anti-invasion and anti-metastasis in NSCLC.

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