microRNA-664 enhances proliferation, migration and invasion of lung cancer cells

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Abstract. Altered microRNA (miR) expression serves an important role in the development and progression of lung cancer. In the present study, the effect of miR-664 on proliferation, migration and invasion of lung cancer cells was assessed. The proliferation of lung cancer cells with an overexpression of miR-664 was examined via MTT assay. The Caspase-Glo3/7 assay was used to examine the effect of miR-664 on cisplatin-induced apoptosis in lung cancer cells. The migration and invasion of lung cancer cells were assessed by Transwell migration and matrigel invasion assays. Western blot analysis was used to examine the protein expression levels. miR-664 improved the proliferation of lung cancer cells and inhibited cisplatin-induced apoptosis of A549 and A427 cells. Furthermore, altered expression of miR-664 affected migration and invasion of lung cancer cells. In addition, a miR-664 mimic decreased E-cadherin expression and increased vementin and Snail expression in lung cancer cells. Notably, the expression level of protein kinase B in A549 cells was changed following altered expression of miR-664. The results of the present study suggest that miR-664 serves an essential role in tumor development and progression in lung cancer.

Introduction

Lung cancer is the most common cause of cancer-related mortality worldwide (1), and is characterized by early distant metastasis, high mortality and poor prognosis. Only 18% of lung cancer patients survive 5 years (2,3). Lung cancer may be subdivided into small-cell lung carcinoma and non-small-cell lung carcinoma (NSCLC). Of NSCLC, 40% of cases are lung adenocarcinoma, which begins in pre-cursor cells that would normally secrete substances such as mucus (4). This type of lung cancer occurs in smokers (54%) and non-smokers (8%) (5). However, it is more common in females than males to develop NSCLC than other types of lung cancer. Surgery is the major treatment for patients with stage I and II NSCLC. Cisplatin-based adjuvant chemotherapy has markedly improved progression-free survival following surgery. Cisplatin has been the most common treatment for patients with stage III and stage IV NSCLC (6). It has been reported that concurrent chemo-radiotherapy may improve the median survival and long-term disease-free survival (7,8). However, its overall response rate is only 30-40%, median survival time is ~9 months and the 1-year overall survival rate is 30-40% (9). Therefore, research efforts have focused on identifying molecular targets and developing molecular-targeted therapies (4,10,11).

microRNAs (miRNAs or miRs) are a family of small non-coding RNAs, which are 18-22 nucleotides in length. Studies have reported that miRNAs serve important roles in tumorigenesis, progression, diagnosis and prognosis of lung cancer. Aberrant miRNA expression, such as miR-15a (12), miR-16 (12), miR-34a (13), miR-34b (14) and miR-125 (15), are important factors affecting the proliferation of lung cancer cells. Ectopic expression of miR-212 influences apoptosis in lung cancer cells (16). Previous studies have reported that miRNAs may be used as prognostic biomarkers in lung cancer: Using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assays, the dynamic change of miRNAs is determined from the serum of patients with NSCLC (17-19).

The expression of miR-664 is increased in certain types of cancer, including lung cancer. However, the mechanism of miR-664 in regulating lung tumorigenesis is unclear (20,21). In the present study, the role of miR-664 in lung cancer cells was examined. Overexpression of miR-664 was demonstrated to promote proliferation, migration and invasion of lung cancer cells. Notably, miR-664 enhanced the epithelial-mesenchymal transition (EMT) of lung cancer cells by targeting protein kinase B (AKT). The results of the present study indicate that miR-664 may be a potential biomarker to predict the prognosis of lung cancer and may be a target for novel therapeutic strategies.

Materials and methods

Cell lines and cell culture. Human lung adenocarcinoma epithelial cell lines A549 and A427 obtained from American

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Type Culture Collection (Manassas, VA, USA) were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.) and penicillin (1 U/ml) and streptomycin (1 mg/ml) at 37˚C and 5% CO_2 in a 95% humidity incubator. All cells were passaged when they reached ~80% confluence.

Transfection. miR-664 mimics (catalogue no. B01001-132) and anti-miR-664 inhibitor (catalogue no. B03001-155) were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). The miR-664 mimic (30 pM) or anti-miR-664 inhibitor (20 pM) was transfected to lung cancer cells using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's protocol, when the A549 and A427 cells reached 70-80% confluence. The scrambled oligonucleotide (B04001-12; Shanghai GenePharma Co., Ltd.) was used as negative control.

RT-qPCR of miR-664. Total RNA was prepared using the RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. Taqman MicroRNA Reverse Transcription kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to make cDNA from the total RNA (200 ng). The PCR conditions were as follows: 95˚C for 5 min, followed by 30 cycles of 95˚C for 30 sec and 60˚C for 30 sec and a dissociation stage. PCR was performed using TaqMan Universal PCR master Mix according to the manufacture's protocol (Thermo Fisher Scientific, Inc.) with a Bio-Rad CFX96 Real-Time PCR machine (Bio-Rad Laboratories, Inc., Hercules, CA, USA). GAPDH was used for RNA quantification, using the $2^{-\Delta\Delta Cq}$ method as descripted previously (22). The sequences of PCR primers used were as follows: 5'-TAC AACACCGGTCACTAACGCATTG-3' and 5'GTATCACCT CCTCCAGCAACTAACA-3' (miR-664 mimic), 5'-CAGTAT CATTCTGTCGCGCTTGTTTA-3' and 5'-TCGGGCGCA ATAATGTAGCGA-3' (miR-664 inhibitor) and 5'-GTCTCC TCTGACTTCAACAGCG-3' and 5'-ACCACCCTGTTGCTG TAGCCAA-3' (GAPDH). All miRNA TaqMan primers were purchased from Ambion; Thermo Fisher Scientific, Inc.

The effect of miR-664 on cell proliferation. An MTT assay (23) was performed to evaluate the effect of miR-664 on the proliferation of lung cancer cells. Either scrambled oligonucleotide or miR-664 mimic was transfected to the two lung cancer cell lines and the cells were grown in 96-well culture plates at a density of $2x10^4$ cells/well. Growth medium (DMEM) containing MTT (20 μl, 5 mg/ml) was added to each well and cultured for 4 h at 37˚C, the medium was changed every other day. Subsequently, 100 μl dimethyl sulfoxide was added to each well and cultured for a further 10 min with gentle agitation at room temperature. The absorbance was measured at 490 nm at room temperature using ELx800 Absorbance Reader (BioTek Instruments, Inc., Winooski, VT, USA). All experiments were performed in triplicate.

The effect of miR-664 on apoptosis. A549 cells overexpressed with either scrambled oligonucleotide or miR-664 mimic were seeded in 24-well culture plates at a density of $2x10^5$ cell/well. Following 12 h of culture, DMEM medium containing 2, 5 and 10 μmol/l cisplatin (Sigma-Aldrich; Merck KGaG, Darmstadt, Germany) was added to each well (according to the concentrations in previous killing curve experiments; data not shown) and culturing continued for a further 72 h at 37˚C and 5% CO_2 in a 95% humidity incubator. The caspase 3/7 activity was examined according to the following protocol: Caspase-Glo reagent (Promega Corporation, Madison, WI, USA) was directly added to each well and incubated for 8 h with gentle agitation at room temperature. The Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific, Inc.) was used to examine the caspase 3/7 activity using 1 min lag time and 0.5 sec/well reads. The experiments were performed in triplicate.

Matrigel invasion assays. To evaluate the effect of miR-664 on migration and invasion of lung cancer cells, a matrigel invasion assay, using invasion and migration chambers purchased from BD Biosciences (Franklin Lakes, NJ, USA) was performed according to the manufacturer's protocol. To evaluate migration, the Transwell insert without matrigel was used. Briefly, either miR-664 mimic or anti-miR-664 inhibitor was transfected to the two lung cancer cell lines and $1x10^4$ cells were seeded to the upper chambers in 0.5 ml DMEM with 0.1% bovine serum albumin. DMEM medium (700 μl) containing 5% FBS was added to the lower chamber. A cotton swab was used to remove the non-invaded cells 12 h later. The invaded cells were stained using Diff Quik solution (Thermo Fisher Scientific, Inc.). Data is expressed as the percent of invasion through the Matrigel Matrix and membrane relative to the migration of cells through the uncoated membrane.

Western blot analysis. The transfected A549 cells were lysed with cold radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China). Then the lysis buffer was mixed with the loading buffer (Beyotime Institute of Biotechnology, Haimen, China) and boiled at 100˚C for 5 min. The protein concentration was assessed using a BCA protein assay kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. SDS-PAGE (10%) was used to separate the proteins (30 μg), prior to being transferred to polyvinylidene fluoride membranes (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Following blocking for 1 h at room temperature in 5% non-fat dry milk in Tris-buffered saline Tween-20 (TBST) buffer, the membranes were washed with TBST buffer and incubated overnight at 4˚C with primary antibodies against the following: B-cell lymphoma-2 (Bcl-2, 1:1,000; sc-7382), Bcl-2 associated X protein (Bax; 1:1,000) (both from Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and E-cadherin (3195; 1:1,000), N-cadherin (13116; 1:3,000), vimentin (5741; 1:3,000), Snail (3897; 1:2,000), AKT (9272; 1:1,000), phosphorylated-AKT (p-AKT; 9271; 1:1,000) and phosphatase and tensin homolog (PTEN; 9559; 1:1,000) (all from Cell Signaling Technology, Inc., Danvers, MA, USA) in TBST with gentle agitation. Following washing with TBST at room temperature three times, the membranes were incubated with anti-rabbit secondary antibody (catalogue no. 7074; 1:3,000) or anti-mouse secondary antibody (catalogue no. 7076; 1:3,000; Cell Signaling Technology, Inc.) at room temperature for ≥60 min with gentle agitation. The Pierce Western Blot Signal Enhancer (Thermo Fisher Scientific, USA) was used

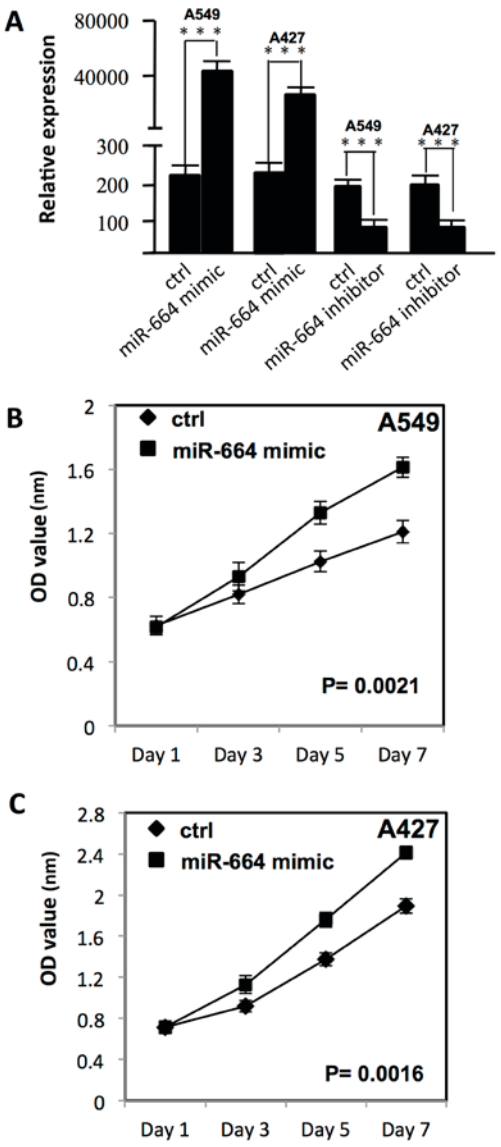

Figure 1. miR-664 decreases the chemo-sensitivity of lung cancer cells. (A) miR-664 expression level was examined using reverse transcription-quantitative polymerase chain reaction on lung cancer cells transfected with either miR-664 mimic or miR-664 inhibitor. ***P<0.05 vs. control. (B) Proliferation of A549 cells following transfection with a miR-664 mimic. (C) Proliferation of A427 cells following transfection with miR-664 mimic. miR, micro RNA; ctrl, control; OD, optical density.

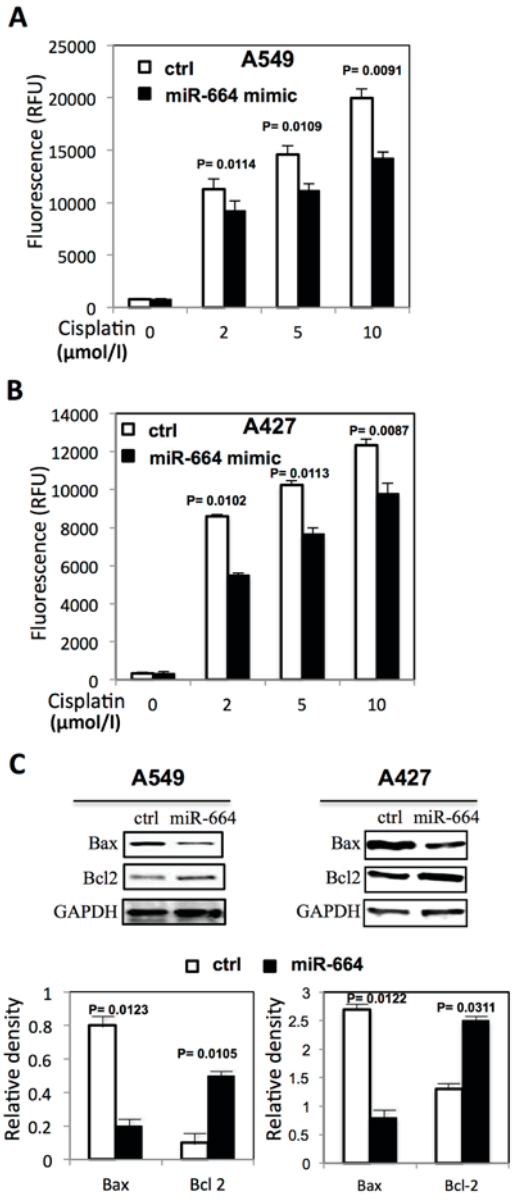

Figure 2. miR-664 decreases apoptosis in lung cancer cells. (A) Caspase 3/7 activity in A549 cells transfected with miR-664 following cisplatin treatment. (B) Caspase 3/7 activity in A427 cells transfected with miR-664 following cisplatin treatment. (C) The level of apoptotic proteins in A549 and A427 cells following cisplatin treatment. Bar graph indicates the relative quantity of proteins on A549 and A427 cells following transfection with miR-664 mimic by densitometric analysis. P<0.05 represents a statistically significant difference vs. control. miR, microRNA; ctrl, control; OD, optical density; RFU, relative fluorescence units; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2 associated X protein.

to develop the immune blot signals. To analyze the relative expression level of each protein, densitometric analysis was performed using ImageLab software version 4.1 (Bio-Rad Laboratories, Inc.). According to the manufacturer's protocol.

Statistical analysis. SPSS version 12.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis by Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-664 promotes the proliferation of lung cancer cells. To examine the effect of miR-664 on the proliferation of A549 and A427 cells, miR-664 was transfected into A549 and A427 cells with Lipofectamine 2000. MTT assay was used to measure the growth curve. As presented in Fig. 1A, following transfection, the miR-664 expression level was significantly increased in A549 and A427 cells (P<0.05). The proliferation of A549 and A427 cells was significantly increased, as compared with the negative control group (P<0.01; Fig. 1B and C).

miR-664 inhibits the apoptosis of lung cancer cells. The function of miR-664 on the apoptosis of A549 cells treated with cisplatin by Caspase-Glo3/7 assay was also evaluated. miR-664 significantly decreased the sensitivity of A549 and A427 cells to cisplatin by inhibiting the caspase 3/7 activities (P<0.05; Fig. 2A and B). The expression of apoptosis-related

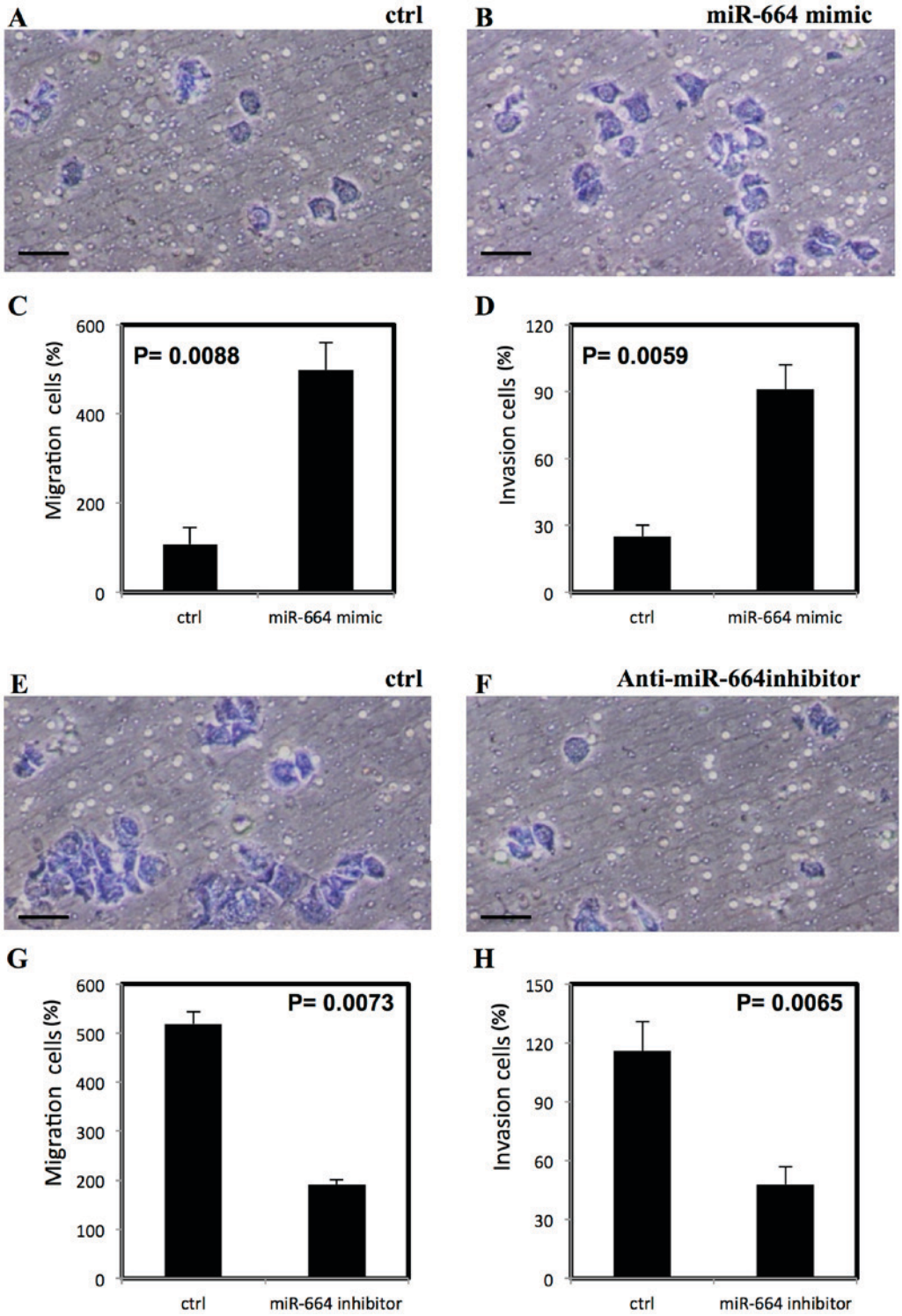

Figure 3. miR-664 increases migration and invasion of A549 cells, stained with Diff Quik stain. (A-D) miR-664 significantly increases migration and invasion of A549 cells. (E-H) Inhibitor of miR-664 significantly decreases the migration and invasion of A549 cells. All experiments were performed in triplicate, P<0.05 represents a statistically significant difference. miR, microRNA. Scale bar=100 μm.

proteins was further analyzed by western blot analysis with or without cisplatin treatment. As presented in Fig. 2C, miR-664 significantly decreased Bax expression (P<0.05) and significantly increased Bcl-2 expression (P<0.05) in A549 and A427 cells.

miR-664 enhances the migration and invasion of lung cancer cells. To examine the effect of miR-664 on migration and invasion of lung cancer cells, Transwell migration and matrigel invasion assays were performed. The miR-664 mimic significantly increased the migration and invasion in A549 cells (P<0.01; Fig. 3A-D) however, the anti-miR-664 inhibitor significantly reduced the migration and invasion of A549 cells (P<0.01; Fig. 3E-H). The significantly increased migration and invasion following treatment with miR-664 mimic was also demonstrated in A427 cells (P<0.01; Fig. 4A-D) and a

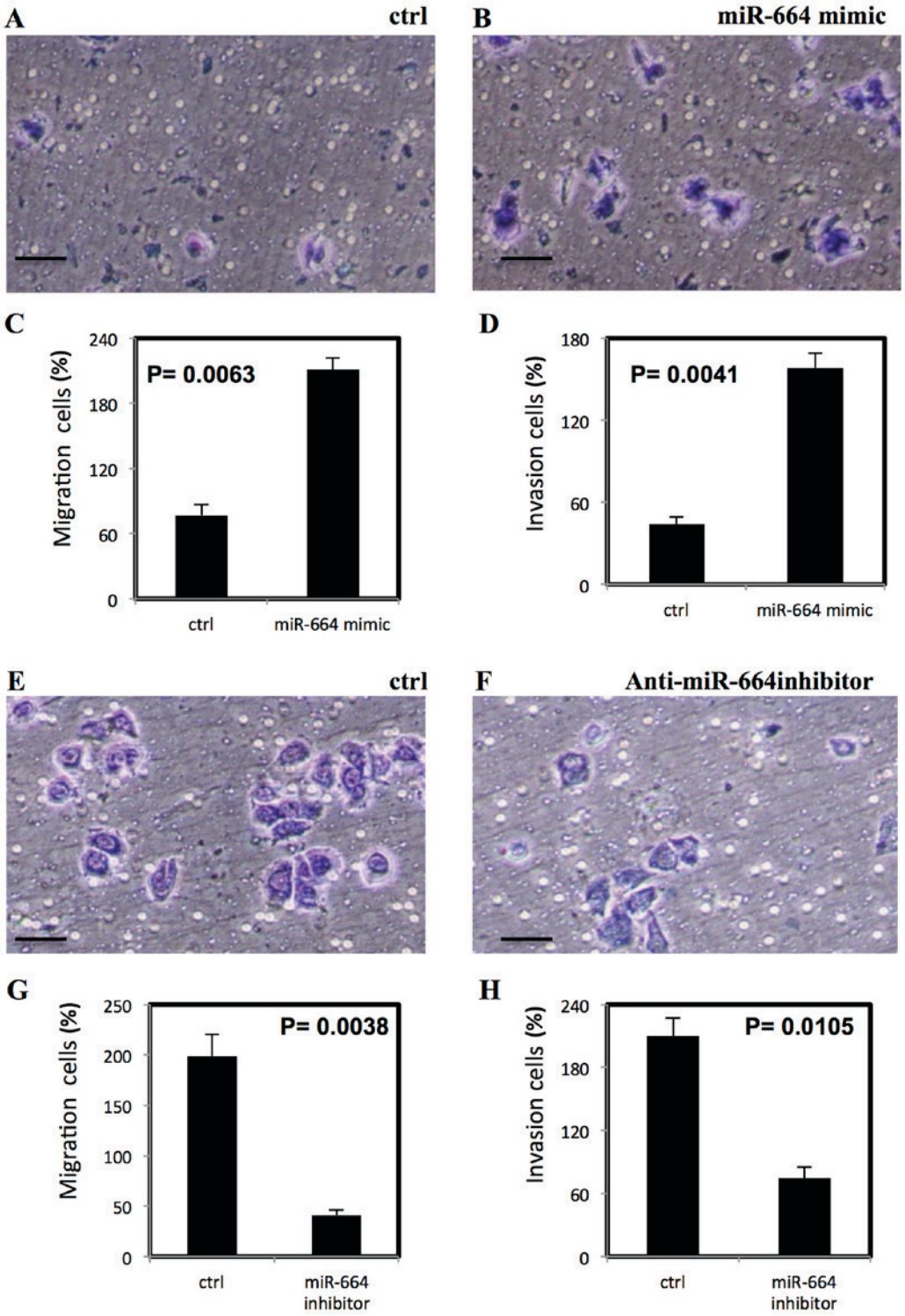

Figure 4. miR-664 increases migration and invasion of A427 cells, stained with Diff Quik stain. (A-D) Significantly increased migration and invasion of A427 cells with miR-664 treatment and (E-H) significantly decreased migration and invasion of A427 cells following treatment with miR-664 inhibitor. All experiments were performed in triplicate, P<0.05 represents a statistically significant difference. miR, microRNA. Scale bar=100 μm.

significant decrease in migration and invasion of A427 cells was observed following treatment with anti-miR-644 inhibitor (P<0.05; Fig. 4E-H).

miR-664 affects EMT-relative proteins expression and activates AKT in A549 cells. The EMT-relative protein expression following transfection of A549 cells with either miR-664 mimic or anti-miR-664 inhibitor was subsequently examined. The E-cadherin expression level was significantly decreased and miR-664 significantly increased the expression of N-cadherin, Snail and vimentin in A549 cells compared with controls (P<0.05; Fig. 5A and B). By contrast, following transfection of A549 cells with anti-miR-664 inhibitor, E-cadherin expression level was significantly increased (P<0.05), and the expression of N-cadherin, snail and Vimentin was significantly decreased (P<0.05; Fig. 5A and B). Notably, the

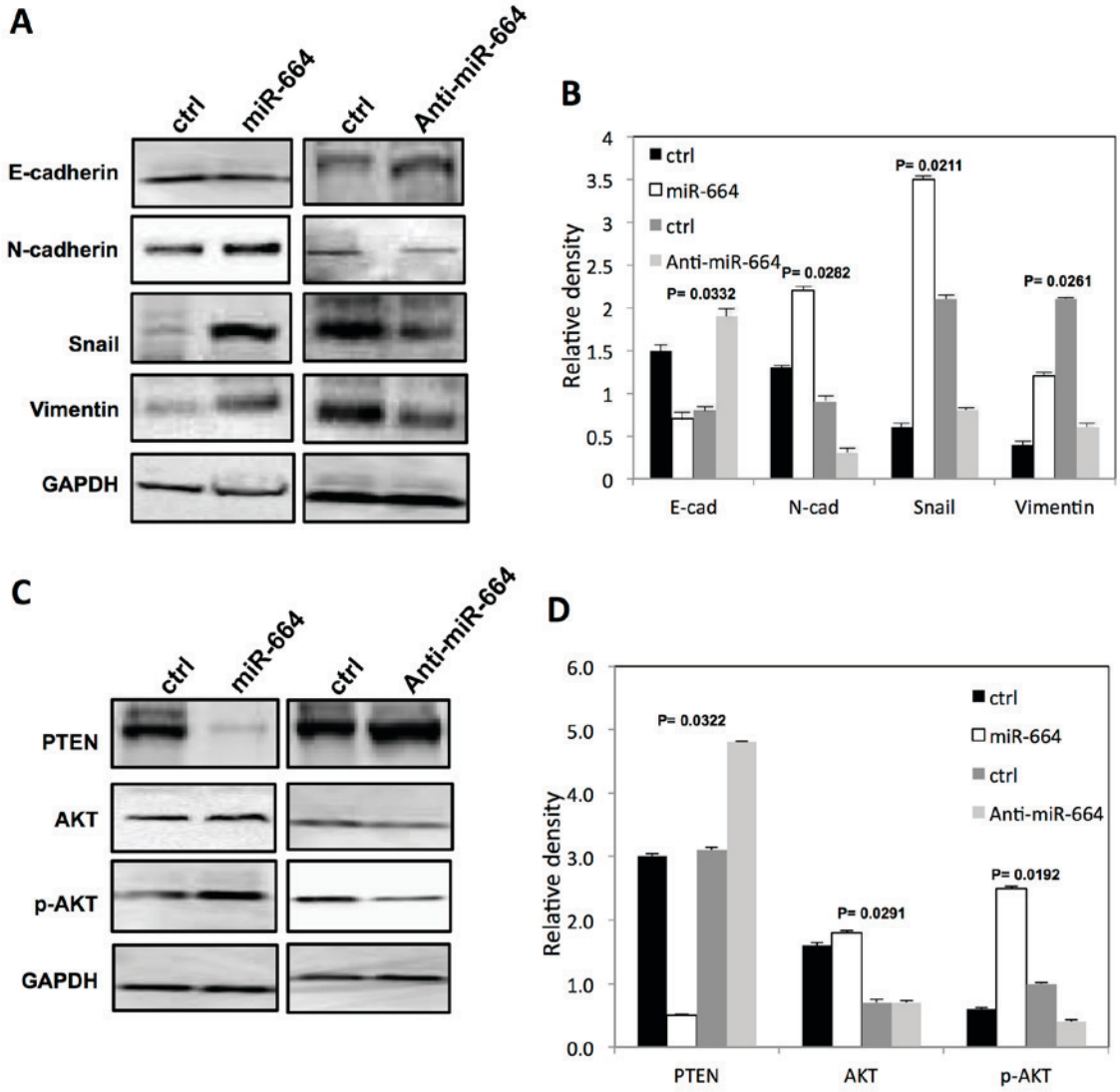

Figure 5. miR-664 regulates expression of EMT markers by activating AKT in lung cancer cells. (A) The level of EMT markers in A549 cells following transfection with either miR-664 mimic or anti-miR-664 inhibitor. (B) Bar graph indicates the relative quantity of proteins in A549 cells following transfection with miR-664 mimic by densitometric analysis. (C) Expression of AKT and PTEN in A549 cells following transfection with either miR-664 mimic or anti-miR-664 inhibitor. (D) Bar graph indicates the relative quantity of proteins in A549 cells following transfection with miR-664 mimic by densitometric analysis. P<0.05 represents a statistically significant difference. miR, microRNA; EMT, epithelial-mesenchymal transition; AKT, protein kinase B; PTEN, phosphatase and tensin homolog; Ctrl, control; E-cad, E-cadherin; N-cad, N-cadherin; p-AKT, phosphorylated-AKT.

miR-664 mimic increased (P<0.05) the expression of p-AKT significantly and decreased (P<0.05) the PTEN expression level in A549 cells significantly (P<0.05; Fig. 5C and D). The downregulation of miR-664 significantly decreased (P<0.05) p-AKT protein expression and increased PTEN expression in A549 cells significantly (P<0.05; Fig. 5C and D).

Discussion

The deregulation of miRNAs has been well studied in numerous diseases, including cancer (24-26). Calin *et al* (27) demonstrated that the expression of miR-15 and miR-16 are decreased in patients with leukemia and may be causally associated with the pathogenesis of chronic lymphocytic leukemia. Another previous study indicated that miR-664 serves an important role in the proliferation and invasion in T-cell acute lymphoblastic leukemia by negatively regulating proteolipid protein 2 (28). Additionally, the expression level of miR-664 is increased in patients with lung cancer (21). In the present study, miR-664 promoted the proliferation of lung cancer cells and miR-664 was demonstrated to decrease cisplatin-induced apoptosis of lung cancer cells. The inhibitory effect on apoptosis was further proved by the increased Bcl-2 expression and decreased Bax expression. These results demonstrate that miR-664 may serve an important role in lung cancer.

The risk of distant metastasis of lung cancer is high and once metastasis occurs, it may become an incurable disease with limited survival time (29). The EMT serves an important role in metastasis and allows epithelial cells to lose their epithelial characteristics and acquire a mesenchymal phenotype (30). It has been demonstrated that miRs regulate EMT in cancer (31). Pacurari *et al* (32) reported that the miR-200 family serves an important role in EMT of lung cancer. The present study demonstrated that miR-664 increased the migration and invasion in A549 cells. By contrast, the

migration and invasion of A549 cells was decreased with the downregulation of miR-664. Furthermore, the present study revealed that miR-664 affected the expression of EMT-relative proteins. Therefore, the present results suggest that miR-664 serves an important role in lung cancer progression.

It has been demonstrated that AKT acts as a survival kinase, and its expression increases in numerous types of cancer, including lung cancer (33). AKT may be activated by a loss of PTEN, which is a well-known tumor suppressor gene in various types of human cancer (34-36). It has been demonstrated that miRNAs contribute to cancer cell growth by regulating PTEN (37). In the present study, miR-664 decreased the expression of PTEN and increased the expression of p-AKT in A549 cells. The results of the present study indicate that miR-664 may regulate proliferation, migration and invasion by regulating the PTEN/AKT signaling pathway.

The present study indicates that miR-664 serves an important role in the regulation of tumorigenesis and malignant progression in lung cancer cell lines. Therefore, miR-664 may be a potential molecular target in lung cancer treatment in the future. Further study it required to examine the expression level of miR-664 in patients with lung cancer.

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