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Overexpression of Muscarinic Receptor 3 Promotes Metastasis and Predicts Poor Prognosis in Non–Small-Cell Lung Cancer

Gengpeng Lin, MD, Longhua Sun, PhD,* Ran Wang, PhD,†, Yubiao Guo, MD,* and Canmao Xie, MD, PhD**

Introduction: Chronic obstructive pulmonary disease (COPD) is an independent risk factor for lung cancer development, but the mechanism is not fully understood. Muscarinic receptor 3 (M3R) has been found to be involved in the progression of small-cell lung cancer and the pathological process of COPD. We hypothesized that M3R may contribute to lung cancer development, especially in patients with COPD.

Methods: The correlation between M3R expression and clinical features of non–small-cell lung cancer (NSCLC) was evaluated in 148 paraffin-embedded archived NSCLC specimens with the use of immunohistochemistry. M3R agonist and siRNA treatments were used to study the role of M3R in NSCLC cell lines. Western blotting and zymography were used to examine the impact of M3R on the PI3K/Akt/matrix metalloproteinase 9 signaling pathway.

Results: The expression of M3R in NSCLC was significantly increased and correlated with tumor metastasis and poor survival of NSCLC patients. NSCLC patients with COPD showed higher expression of M3R than those without COPD ($p = 0.0014$). Moreover, M3R expression was inversely related to percent forced expiratory volume in 1 second ($r = 0.7017$, $p < 0.0001$) and forced expiratory volume in 1 second /forced vital capacity ($r = 0.5057$, $p < 0.0001$), but positively related to smoking history. Down-regulation of M3R resulted in the inhibition of migration and invasion ability of NSCLC cell lines A549 and L78. Furthermore, M3R enhanced the expression and activity of matrix metalloproteinase 9 through PI3K/Akt, which promoted the migration and invasion of NSCLC cell lines.

Conclusion: Our results suggest that overexpression of M3R in NSCLC promotes the progression of NSCLC, which could contribute to lung cancer development in COPD patients. M3R could be another pharmacological target in lung cancer, especially in COPD patients.

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Lung cancer is the leading cause of cancer-related death in the world and approximately 80% to 85% of lung cancers are non–small-cell lung cancer (NSCLC).[1] The prevalence and mortality of lung cancer have been increasing dramatically in recent years, not only in developing countries like China, but also in developed countries of Europe.[2] Lung cancer will overtake breast cancer and become the leading cause of death in women.[2] The leading cause of death from lung cancer is metastasis. Often, metastasis already occurs even before the diagnosis of lung cancer is made, leading to recurrence and treatment failure in lung cancer patients.

Chronic obstructive pulmonary disease (COPD) is characterized by progressive obstruction of the airway, which is not fully reversible after treatment with bronchodilators.[3] COPD patients are at great risk for developing lung cancer.[4] Multiple studies have shown that individuals with emphysema and airway obstruction have an increased risk for the development of lung cancer and a worse prognosis.[5] And lung cancer screening is necessary in COPD patients.[6] However, the mechanism of COPD in promoting lung cancer development is not well understood.

Muscarinic receptor 3 (M3R) is one of the five muscarinic receptors (M1R-M5R)[7] which plays an important role in many pathological processes of lung disease, including COPD. And the use of drugs targeting M3R has become one of the basic therapies for COPD.[8–11] Recent studies have shown that M3R is a major player in many kinds of cancer, like colon cancer, gastric cancer, breast cancer, and lung cancer.[12–15] It has been shown that acetylcholine (Ach) promotes proliferation and cell migration of small-cell lung cancer (SCLC) through M3R.[16,17] Our previous study also confirmed the role of M3R in enabling cell adherence to the extracellular matrix.[18] However, the role of M3R in NSCLC progression has not been fully investigated. We hypothesized that overexpression and activation of M3R may contribute to lung cancer development and metastasis in patients with COPD. To test this hypothesis, we examined the expression of M3R in NSCLC tissues and explored the relationship between the intensity and clinical–pathological features such as metastasis status and the 5-year

survival rate of NSCLC patients. We also used an M3R agonist and siRNA to study the influence of M3R on the migration and invasion behavior of NSCLC cell lines. Finally, we verified which signal pathway could be responsible in this process.

MATERIALS AND METHODS

Patient Information and Tissue Specimens

Paraffin-embedded archived NSCLC samples were obtained from 148 patients diagnosed with NSCLC between January 2000 and December 2007 in the Department of Pathology at the First Affiliated Hospital of Sun Yat-sen University. Of these, 60 cases were diagnosed with COPD and 88 cases were not diagnosed with COPD. The histological characterization and clinical–pathological staging of the samples was determined according to World Health Organization criteria[19] and the current International Union Against Cancer tumor-node-metastasis classification[20] or International Association for the Study of Lung Cancer tumor-node-metastasis classification. The diagnosis of COPD was made according to Global Initiative for Obstructive Lung Disease.[21] Detailed clinical information for all patients is summarized in Supplementary Table 1 (Supplemental Digital Content 1, http://links.lww.com/JTO/A514).

Cell Lines and Cell Culture

Human lung adenocarcinoma cell lines A549, PC9, SPC-A1, and GLC82, human squamous lung cancer cell line L78, and human lung fibroblast (HLF) cell line were used in this study. Among them, A549, PC9, and HLF were obtained from Cell Bank, Chinese Academy of Sciences (Shanghai, China) and kept in our laboratory. SPC-A1, GLC82, and L78 were generously provided by Prof. Liantang Wang from Department of Pathology in the First Affiliated Hospital of Sun Yat-Sen University. Cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, BRL, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gibco, BRL) at 37°C in a humidified incubator with 95% air and 5% CO_2.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated from the cells using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction was carried out using the FastStart Universal SYBR Green Master(ROX, Roche, Toronto, Ontario, Canada) on the ABI StepOne quantitative real-time polymerase chain reaction detection system. Beta-actin was used as reference gene. The sequences of the primer pairs were as follows: M3R, 5'-GCCAAACGAACAACAAAGAGAG-3' (F) and 5 '-CCAACAAAGTATTGCCAG AACA-3' (R); β-actin, 5'-CGCCAGCTCACCATGGATGATGAT-3' (F) and 5'-TCTCTTGCTCTGGGCCTCGTCG-3' (R). Each sample was tested in triplicate and the mean expression level was calculated.

Western Blot Analysis

Total protein was extracted from cells with a lysis buffer. Protein concentration was measured using the BCA Protein Assay kit (CWBiotech, Beijing, China). Equal amounts of protein were separated by sodium dodecyl sulfate (SDS) polyacrylamide (10% gel). After electrophoretic transfer to a polyvinylidene difluoride membrane, nonspecific binding sites were blocked with 5% nonfat milk in Tris-buffered saline Tween-20 (TBST) (0.1% Tween 20) followed by incubation with primary antibodies overnight at 4°C. After three rinses with TBST, anti-rabbit HRP secondary antibody (1:5000) or antimouse horseradish peroxidase (HRP) secondary antibody (1:5000) was added. Protein bands were detected using enhanced chemiluminescence (Millipore, Billerica, MA).

Immunohistochemistry

Immunohistochemistry (IHC) studies were performed using a standard streptavidin-biotin-peroxidase complex method (EnVision™ Detection Systems, Dako, Copenhagen, Denmark). Slides were incubated with polyclonal rabbit anti-human M3R (1:300, Santa Cruz, Dallas, TX) overnight at 4°C. A semiquantitative scoring criterion for IHC of M3R was used, in which both staining intensity and positive areas were recorded. A staining index (values 0–12), obtained as the intensity of M3R positive staining (negative = 0, weak = 1, moderate = 2, or strong = 3 scores) and the proportion of immune-staining positive cells of interest (< 25% = 1, 25%–50% = 2, > 50%– < 75% = 3, ≥75% = 4 scores) were calculated. Two independent pathologists (RW and MZ) blinded to clinical–pathological information performed the scorings. Cutoff values to define high and low expression of M3R were chosen according to a measure of heterogeneity by using log-rank test statistics with respect to overall survival. An optimal cutoff value was identified. A score of more than 6 was used to define tumors with high expression and a score of 6 or lower defined tumors with low expression of M3R.

Cell Proliferation Assay

A549 and PC9 cells were plated on 96-well plates (Corning, NY) and allowed to attach overnight. After 24 hours, 48 hours, and 72 hours, 20 μl of 5 mg/ml methyl thiazole tetrazolium (MTT) (Sigma, St. Louis, MO) solution was added to each well and incubated for 4 hours at 37°C. After the media was removed, 200 μl of DMSO (Sigma) was added and absorbance values at 490 nm were recorded using a microculture plate reader.

Wound-Healing Assay

A549 and L78 cells were grown to confluence in cell culture dishes. A wound was inflicted on the monolayer by using a pipette tip. Cells were then serum starved and allowed to migrate for 24 hours.

Cell Migration and Invasion Assays

Cell migration assays were performed using a 24-well transwell chamber with an 8.0 μm pore polycarbonate membrane insert (Corning). For the invasion assay, the inserts were coated with 40 μl Matrigel (1:8 dilution; BD Bioscience, Bedford, MA) Cells (1×10^5) in 0.5 ml of serum-free media were placed in the upper compartment. The lower chamber was filled with 600 μl of media supplemented with 10%

fetal bovine serum. After incubation for 24 hours, the cells on the upper surface of the membrane were removed and the migrated cells on the lower surface were fixed with methanol and stained with crystal violet. Numbers of invaded cells were counted in 10 randomly selected high-power fields under a microscope. These experiments were performed in triplicate.

siRNA treatment

Cells were seeded in a 6-well plate 24 hours before transfection. The siRNAs were transfected at a concentration of 50 nmol/liter each with RNAiMax (Invitrogen) according to the manufacturer's instructions. After transfection, mRNA and protein levels were assessed 24 hours and 48 hours later, respectively. The target sequences of M3R siRNAs are as follows: 5'- CGAGCCAAACGAACAACAA -3' (si 1) and 5'-CCTGGTAATTGTGTCATTT-3' (si 2).

Zymography

Cells were cultured in fresh RPMI 1640 medium for another 24 hours after treatment. Culture media was then collected and centrifuged, and the supernatant was preserved. Before measuring the protein concentration using the BCA kit, the culture medium was concentrated using ultrafiltration (Millpore, Billerica, MA). Protein (20 ug) was loaded and separated using a 10% SDS polyacrylamide gel containing 1% gelatin at 27 mA/gel and 4°C. The gel was then washed, incubated, stained, and destained following the instructions of the gelatin zymography kit (Applygen, Beijing, China).

Statistical Assay

Data are expressed as mean ± standard deviation from three independent experiments. Comparisons among groups were performed using Student's *t* test or one-way analysis of variance. The χ^2 test and Spearman analysis were used to analyze the relationship between M3R expression and clinical–pathological characteristics. Spearman analysis was used to examine the relationship between M3R expression and lung function. Kaplan–Meier method was performed to plot survival curves whereas the log-rank test was used to compare survival curves. Univariate and multivariate Cox regression analyses test were also performed to identify possible risk factors. A value of p less than 0.05 was considered statistically significant. Statistical tests were performed using SPSS 13.0.

RESULTS

Increased Expression of M3R in NSCLC Cell Lines and Tissues

Western blotting was used to determine M3R protein expression both in human NSCLC cell lines, including A549, PC9, SPC-A1, GLC82, and L78, and a human lung fibroblast cell line HLF. All NSCLC cell lines demonstrated notably higher levels of M3R expression compared with HLF (Fig. 1*A*). Furthermore, comparative analysis of M3R expression was conducted on 10 pairs of matched NSCLC tissue and adjacent noncancerous tissue. The expression of M3R protein in eight NSCLC samples was much higher than in the paired adjacent noncancerous tissue (Fig. 1*B*). The specificity of the M3R antibody used in the study is shown in Supplementary Figure 1 (Supplemental Digital Content 2, http://links.lww.com/JTO/A515).

Association between Increased Expression of M3R and Progression of NSCLC

To further examine whether increased expression of M3R was related to the progression of NSCLC, 148 cases of archived paraffin-embedded samples were assayed using IHC. The mean age of the 148 NSCLC patients was 64.4 years (range, 24–86 years), and the follow-up period was from 0.7 to 90 months, with a median of 21.3 months. A total of 114 deaths were reported during the follow-up period.

The results of IHC staining are summarized in Supplementary Table 2 (Supplemental Digital Content 1, http://links.lww.com/JTO/A514). M3R was highly expressed in 85 of 148 human NSCLC samples (57.4%), with no significance observed among the three different subtype groups ($p = 0.081$), namely squamous cell carcinoma, adenocarcinoma, and other subtypes such as large cell carcinoma. Moreover, the expression level of M3R correlated with the progression of NSCLC. Statistical analyses showed no relationship between patient age or sex and M3R expression. However, M3R expression strongly correlated with clinical stage ($p = 0.003$), N classification ($p = 0.009$), and M classification ($p = 0.001$) of NSCLC patients. Spearman analyses also revealed a correlation between M3R expression level and clinical stage ($p < 0.001$), N classification ($p < 0.001$), and M classification ($p < 0.001$).

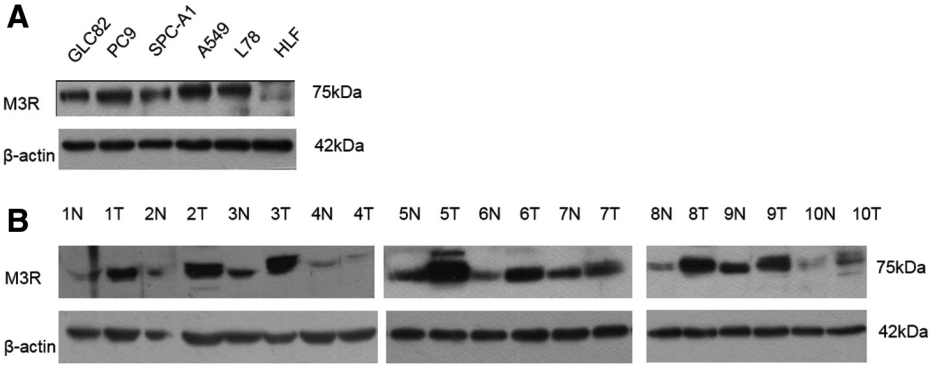

FIGURE 1. M3R is overexpressed in both NSCLC cell lines and tissues. *A* Western blot analysis of M3R expression in NSCLC cell lines (GLC82, PC9, SPC-A1, A549, and L78) and HLF. *B*, Western blot analysis of M3R protein level in 10 pairs of tissues (T: tumor, N: adjacent noncancerous lung tissue). β-actin was used as a loading control. M3R, muscarinic receptor 3; NSCLC, non–small-cell lung cancer; and HLF.

Association between M3R Expression and Prognosis for NSCLC Patients

Statistical analysis revealed an inverse correlation between M3R expression level and the NSCLC patient cumulative 5-year survival rate. The cumulative 5-year survival rate of patients with low M3R expression was 39.3% (95% confidence interval [CI]: 27.15%–51.45%), whereas the 5-year survival of those patients with high M3R expression was 12.9% (95% CI: 5.84%–19.96%). We next explored the role of M3R in predicting the prognosis of NSCLC patients with or without COPD. In the COPD group, patients with a high level of M3R had a survival rate of 9.4% (95% CI: 4.30%–14.50%), which was significantly lower than the survival rate of those patients with a low level of M3R (38.5%, 95% CI: 27.72%–38.39%). In patients without COPD, a high level of M3R also predicted a poorer 5-year survival rate (16.5%, 95% CI: 10.03%–22.97%) than did a low level of M3R (39.9%, 95% CI: 30.69%–49.11%; Fig. 2*B*).

M3R expression, clinical stage, TMN classification, age, sex, and lung function status (with and without COPD) were analyzed by using univariable and multivariable Cox regression analyses. Univariable analyses revealed that clinical stage, M3R expression, TMN classification, and lung function status were significant predictors of NSCLC. Multivariable analysis showed that clinical stage, M3R expression, and lung function status were independent predictors of NSCLC prognosis (Table 1).

Expression Level of M3R is Closely Related to Smoking History and Airway Obstruction Status

NSCLC patients with COPD had a higher level of M3R expression (Fig. 3*A*). This prompted us to investigate the relationship between M3R expression and lung function. On the basis of a lung function test, 60 of 148 NSCLC patients were diagnosed with COPD, with an average precent forced expiratory volume in 1 second (%FEV1; of the predicted value) of 61.7±19.8%, and an average FEV1/forced vital capacity (FVC) of 53.6±11.9%. Correlation analysis showed that the M3R expression level was conversely related to %FEV1 ($r = 0.7017$, $p < 0.0001$) and FEV1/FVC ($r = 0.5057$, $p < 0.0001$; Fig. 3*B*, *C*). We next explored the relationship between M3R expression and smoking history. M3R expression positively correlated with smoking history ($r = 0.3169$, $p = 0.0014$; Fig. 3*D*, *E*).

Role of M3R in the Growth of NSCLC Cell Lines

In light of a previous study showing that M3R promotes cell growth in SCLC, we first analyzed M3R-induced cell growth in A549 and L78 cells. A549 and L78 were treated with M3R siRNA. The proliferation rate was determined by the MTT assay. Little reduction was observed in the proliferation rate of A549 and L78 cells after siRNA treatment ($p < 0.01$; Fig 4*B*).

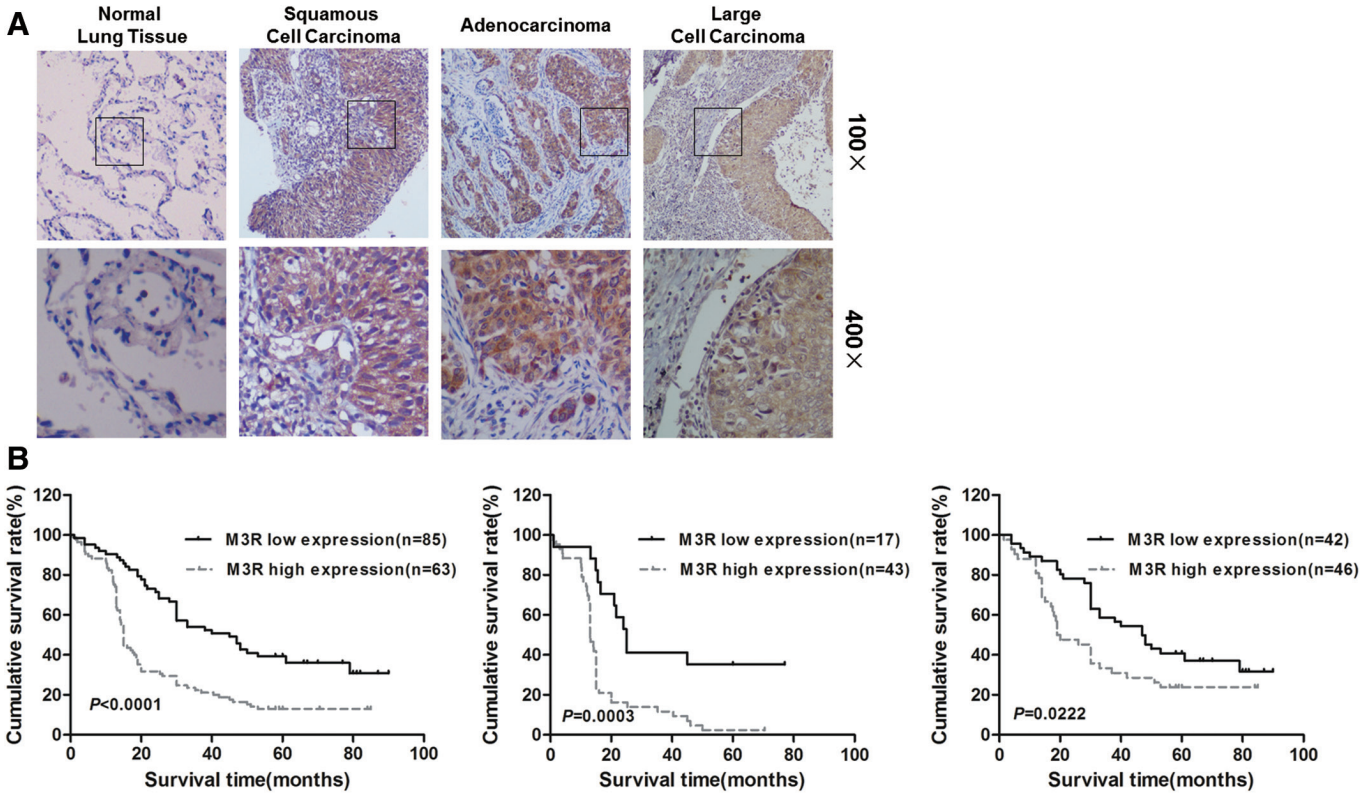

FIGURE 2. Overexpression of M3R correlates with poor prognosis and metastasis in NSCLC patients. *A,* Representative immunohistochemistry pictures of M3R expression in different NSCLC subtypes and normal lung tissues. *B,* Survival curves of NSCLC patients with high or low M3R expression ($n = 148$, $p < 0.0001$; left panel). Differences within subgroups of NSCLC with COPD ($n = 60$, $p = 0.0003$; middle panel) and NSCLC without COPD ($n = 88$, $p = 0.0222$; right panel). M3R, muscarinic receptor; 3 NSCLC, non–small-cell lung cancer; COPD, chronic obstructive pulmonary disease.

TABLE 1. Univariate and Multivariate Analysis of Different Prognostic Parameters in Patients with Lung Cancer by Cox Regression Analysis (Forward Conditional)

		Univariate Analysis				Multivariate Analysis			
	Assigned Score	*p*	SE	HR	95%CI	*p*	SE	HR	95%CI
T classification		0.002	0.094	1.342	1.116–1.614				
I	1								
II	2								
III	3								
IV	4								
N classification		<0.001	0.076	1.539	1.325–1.787				
0	0								
1	1								
2	2								
3	3								
M classification		<0.001	0.233	3.947	2.498–6.234				
0	0								
1	1								
TMN stage		<0.001	0.100	1.880	1.547–2.285	<0.001	0.101	1.756	1.441–2.139
I	1								
II	2								
III	3								
IV	4								
M3R expression		<0.001	0.199	2.398	1.623–3.545	0.001	0.206	1.987	1.327–2.974
Low	0								
High	1								
Lung function		<0.001	0.192	2.194	1.506–3.198	0.002	0.196	1.839	1.253–2.698
With COPD									
Without COPD									
Age, yrs		0.156	0.209	1.345	0.893–2.026				
≤60	0								
>60	1								
Sex		0.285	0.238	1.289	0.809–2.055				
Female	0								
Male	1								

SE, standard error; HR, hazard ratio; CI, confidence interval; M3R, muscarinic receptor; 3 TNM, tumor-node-metastasis; COPD, chronic obstructive pulmonary disease.

Role of M3R in Cell Invasion and Migration of NSCLC Cell Lines

Next, we investigated the role of M3R in the invasion and migration abilities of NSCLC cell lines. Activation of M3R with Ach significantly increased cell invasion into Matrigel-coated transwell membranes (Supplementary Figure 2B, Supplemental Digital Content 3, http://links.lww.com/JTO/A516), whereas down-regulation of M3R with siRNA resulted in reduced cell invasion ($p < 0.01$; Fig. 4*D*). Similar results were also observed in wound-healing assays, where M3R agonist promoted cell migration in a scratch assay on monolayer-cultured cells (Supplementary Figure 2A, Supplemental Digital Content 3, http://links.lww.com/JTO/A516; Fig. 4*C*). Taken together, these results suggest that M3R induces cell invasion and migration of NSCLC cell lines.

M3R Promotes Invasion and Migration through PI3K/Akt/Matrix Metalloproteinase 9

To further investigate the molecular mechanism by which M3R promotes invasion and migration, levels of metastasis-related genes matrix metalloproteinase (MMP9) and E-cadherin were examined. Using zymography and Western blot, we demonstrated that activation of M3R resulted in the up-regulation of MMP9 expression and activity, and down-regulation of E-cadherin (Fig.5*C*, *D*; Supplementary Figure 2C, D, Supplemental Digital Content 3, http://links.lww.com/JTO/A516), whereas down-regulation of M3R attenuated the expression and activity of MMP9 and up-regulated E-cadherin (Fig. 5*A*, *B*). Because Akt is an important factor in the metastatic process for regulating MMP proteins and epithelial-mesenchymal (EMT)-related genes like E-cadherin, we investigated the relationship between M3R and Akt. Activation of M3R increased the level of p-Akt, whereas down-regulation of M3R decreased

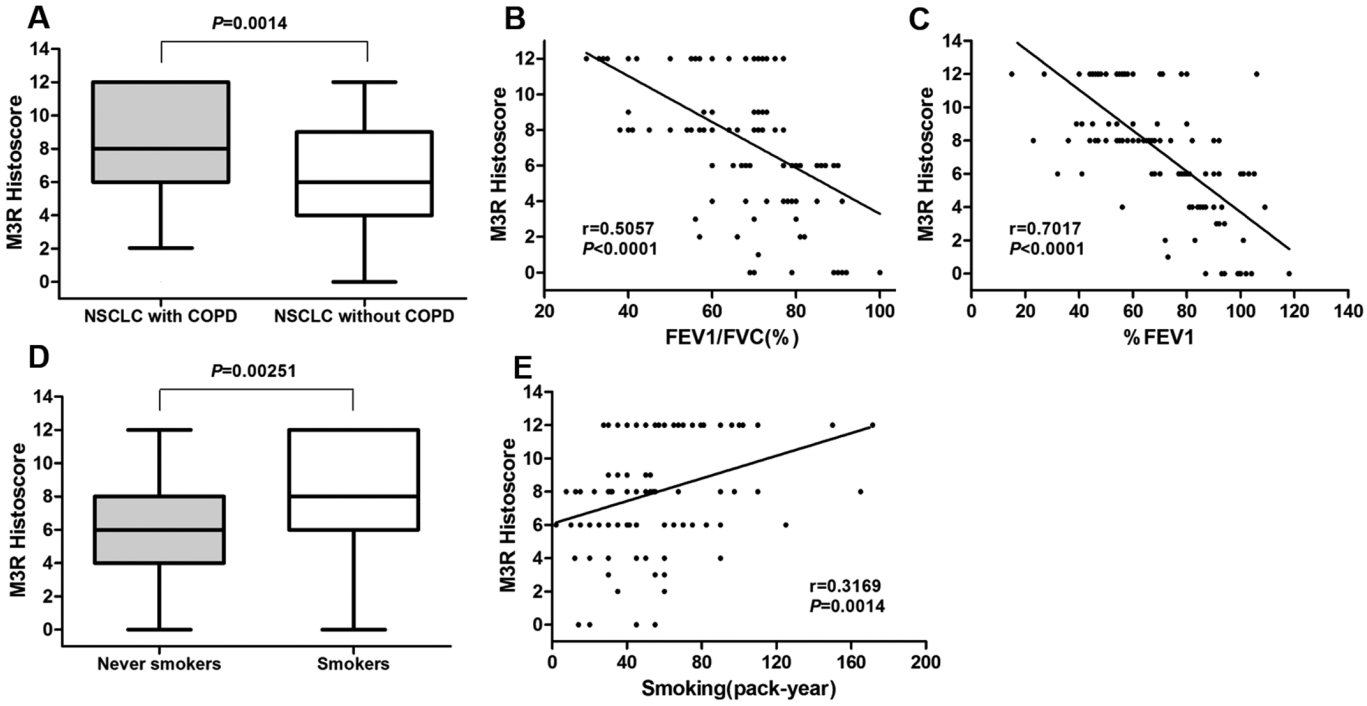

FIGURE 3. Expression of M3R in NSCLC is associated with airway obstruction and smoking. *A*, Differences in M3R expression in NSCLC tissues between patients with and without COPD. *B*, Inverse correlation between M3R expression and FEV1/FVC ($r = 0.5057$, $p < 0.0001$). *C*, Inverse correlation between M3R expression and %FEV1 ($r = 0.7017$, $p < 0.0001$). *D*, M3R expression levels in NSCLC patients with and without a smoking history. *E*, Correlation between M3R expression and smoking (pack-years) ($r = 0.3169$, $p = 0.0014$). M3R, muscarinic receptor 3; NSCLC, non–small-cell lung cancer; FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity; COPD, chronic obstructive pulmonary disease.

p-Akt. Using the specific PI3K/Akt inhibitor, LY294002 (CST, Danvers, MA), we found that Ach-promoted invasion and migration was abolished and the activity and expression of MMP9 levels decreased, whereas E-cadherin expression was slightly increased (Fig.5*C*, *D*). Furthermore, we used wound-healing and transwell assays to explore the effect of M3R activation and Akt inhibition via LY294002 on migration and invasion of A540 and L78 cells. Migration and invasion, which were promoted by an Ach concentration of 10^{-4}M, was abolished by LY294002 (Fig.5*E*, *F*). These results demonstrate that M3R promotes invasion and migration through the PI3K/Akt pathway.

DISCUSSION

This study describes the role of M3R in the progression of NSCLC, especially in the context of COPD. To our knowledge, this is the first study to demonstrate that M3R promotes migration and invasion of NSCLC, and that high expression of M3R is an indicator of poor prognosis in NSCLC. Moreover, the expression level of M3R is significantly higher in NSCLC patients with COPD compared with those in patients without COPD. Furthermore, the M3R expression level correlates inversely with %FEV1 and FEV1/FVC, two important indices for evaluating the severity of COPD. Further, we investigated the molecular mechanism through which M3R mediates migration and invasion of NSCLC.

M3R, a G protein–coupled receptor, belongs to the family of muscarinic receptors (M1R–M5R).[7] M3R is involved in the pathological process of COPD, including airway constriction,[9] sputum production,[10] and macrophage infiltration.[22] Inhaled M3R antagonists like tiotropium, have become the cornerstone in the treatment of COPD.[3] Recently, M3R was found to be involved in the development of cancer. Previous work from our group and others has suggested that M3R promotes cell proliferation and migration of SCLC cell lines, and that such promotion can be inhibited by M3R antagonists.[16,18] However, the role of M3R in the progression of NSCLC remains unclear. In this study, we address this gap in knowledge, and demonstrate that M3R promotes migration and invasion, but not proliferation of NSCLC. NSCLC cell lines treated with an M3R agonist demonstrate more aggressive behavior, however, down-regulation of M3R does not significantly inhibit cell proliferation of NSCLC cell lines. These results were further confirmed by the correlation analysis between M3R expression level and NSCLC progression in 148 patients. The expression level of M3R correlates with the metastasis status of NSCLC patients, but not with the tumor size. Even though M3R promotes progression of both SCLC and NSCLC, the mechanism of the different roles of M3R in cell growth among SCLC and NSCLC is still unclear. One possible explanation is that different histopathological subtypes of lung cancer arise from different cells of origin,[23] and M3R may function differently among these cells. Nevertheless, the real situation in the patients with lung cancer may be different. Further research is needed to clarify the differences between the roles of M3R in SCLC and NSCLC and we look forward to the results of clinical trials to test the use of M3R antagonists in COPD patients with lung cancer.

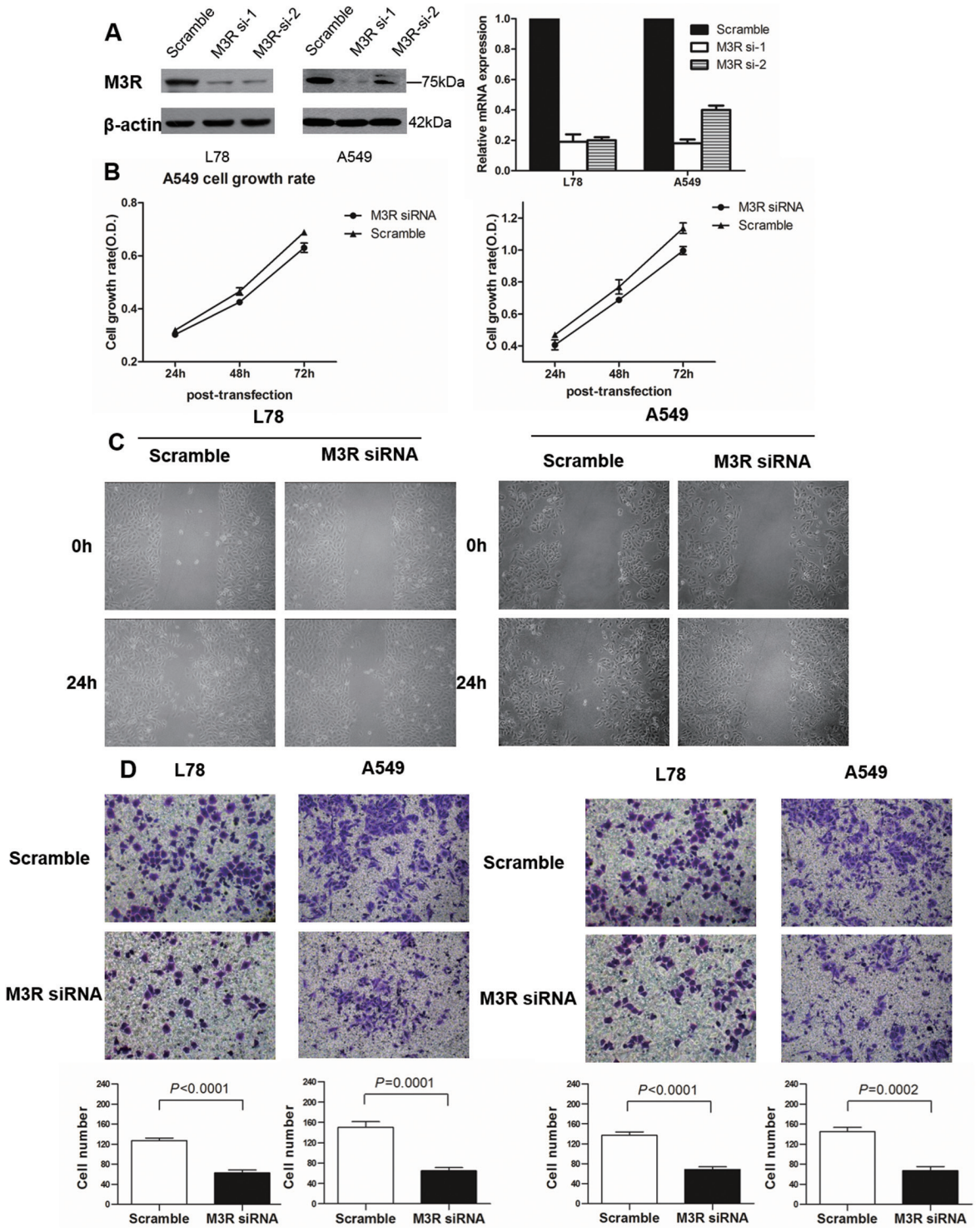

FIGURE 4. M3R promotes proliferation, migration, and invasion of non–small-cell lung cancer cell lines. *A,* Knockdown efficiency of M3R siRNA in A549 and L78 cells. *B,* Growth curves of L78 and A549 after M3R siRNA treatment. A small reduction of absorbance was observed after siRNA treatment ($p < 0.01$). *C,* Wound-healing assay to analyze cell migration after M3R siRNA treatment. *D,* Migration and invasion assay using a transwell system (representative images of migration chambers; average counts from 5 random microscopic fields). M3R siRNA inhibited the migration and invasion ability of L78 and A549. M3R, muscarinic receptor 3.

COPD is a risk factor and an indicator of worse prognosis of lung cancer. Patients with COPD have a 10-fold higher risk of developing lung cancer.[4] Further, lung cancer patients with COPD have a significantly shorter survival time compared with those without COPD.[5] This group of patients tends to have more rapid cancer progression. The underlying mechanism may

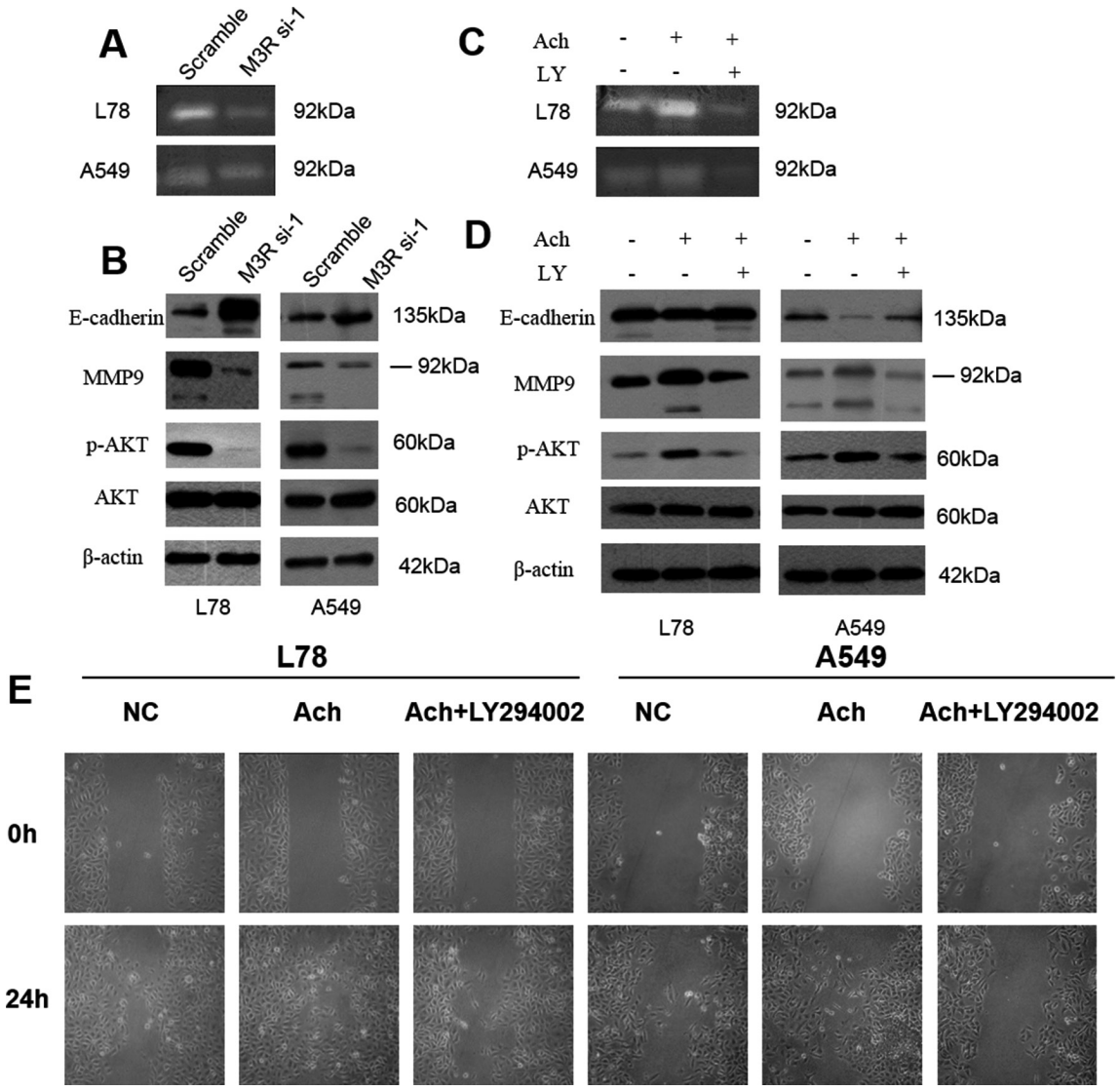

FIGURE 5. M3R promotes the migration and invasion of non–small-cell lung cancer cell lines through the PI3K/Akt/MMP9 pathway. *A*, Zymography of MMP9 in conditioned medium of cells treated with M3R siRNA. *B* and *D*, Western bot assay of p-Akt, Akt, E-cadherin, and MMP9 in indicated cells. *B*, Knockdown of M3R results in the reduced expression of p-Akt and MMP9, but elevated expression of E-cadherin. *D*, Activation of M3R by Ach induces expression of MMP9 and p-Akt, but reduces expression of E-cadherin. PI3K inhibitor abolishes the effect of Ach. *C*, Zymography of MMP9 using conditioned medium from cells treated with Ach and LY294002. *E*, Wound-healing assay for cells migration ability after Ach and LY294002 treatment. A concentration of 10^{-4}M Ach promotes migration, but migration is abolished by LY294002. *F*, Migration and invasion assay using a transwell system (representative images of migration chambers; average counts from 5 random microscopic fields). Cells were treated with 10^{-4}M Ach with and without 20μM LY294002, respectively. A concentration of 10^{-4}M Ach promotes migration while LY294002 treatment inhibits it. M3R, muscarinic receptor 3, Ach, acetylcholine; MMP9, matrix metalloproteinase 9; NC, negative control; pAKT, phosphor-AKT.

include chronic inflammation, oxidative stress, and genetic predisposition.[24] However, none of these have been explored in depth. A recent study has suggested that hypoxia might promote tumor growth, and, thus, predispose COPD patients to lung cancer development.[25] In our study, we found that lung cancer patients with COPD have a shorter survival time than those without COPD. The 5-year survival rate of lung cancer patients with COPD is only 11.7% (95% CI: 3.66%–19.7%), whereas the survival rate of those without COPD is 32.7% (95% CI: 22.9%–42.5%). Also, lung cancer patients with COPD have a higher expression level of M3R, which promotes migration and invasion of NSCLC. Thus, M3R could be involved in the development of lung cancer in patients with COPD. However, why M3R is highly expressed in NSCLC patients with COPD is still unclear. Recent studies suggested that smoking and inflammatory factors could be responsible. It has been reported that cigarette smoke extract increases the expression of M3R in 16HBE cell line, on both mRNA and protein levels.[26] Tumor necrosis factor-α, an inflammatory factor elevated in COPD patients,[27] was found to enhance the expression of M3R.[28]

In our study, we found that M3R expression correlates with severity of airway obstruction and smoking. Chronic irritants, such as cigarette smoke, are pathologic factors of COPD, which causes chronic inflammation.[3] Therefore, one possible explanation is that smoking causes M3R overexpression and inflammation in COPD patients, both of which induce airway obstruction. Because inhaled M3R antagonist has been widely used in COPD, M3R could be another therapeutic target for the prevention and treatment of lung cancer.

M3R has been found to mediate different signal pathways in different kinds of cancer.[12] In SCLC cell lines, M3R regulates cell proliferation via the mitogen-activated protein kinase (MAPK) pathway.[16] M3R also regulates the activity of E-cadherin and integrins, key factors in cell adhesion and migration of SCLC cell lines.[29] In our study, we first examined whether M3R regulates metastasis-related genes like MMP9 and E-cadherin. Knockdown of M3R using siRNA resulted in a notable decrease of MMP9 and an increase of E-cadherin. This result was further verified by M3R agonist treatment. Next, we explored how M3R regulates these metastasis-related genes. The PI3K/Akt pathway, which has been reported to regulate the transcription of MMPs and EMT-related genes,[30,31] was studied. Knockdown of M3R using siRNA leads to a decrease of p-Akt, without affecting total Akt. This suggests that M3R promotes invasion and migration of NSCLC through Akt.

In conclusion, our study shows that M3R induces invasion and migration of NSCLC through Akt. M3R expression is higher in NSCLC with COPD. This implies that M3R could take part in the development of lung cancer in patients with COPD. Thus M3R antagonists could be useful in the treatment and prevention of lung cancer in COPD patients.

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