ADAM15 targets MMP9 activity to promote lung cancer cell invasion

DAN-DAN DONG[1], HUI ZHOU[2] and GAO LI[3]

[1]Department of Pathology, Sichuan Academy of Medical Sciences, Sichuan Provincial People's Hospital, Chengdu, Sichuan 610072; [2]Department of Thoracic Medicine, The Affiliated Cancer Hospital of Xiangya School of Medicine, Central South University, Changsha, Hunan 410013; [3]Department of Thoracic Surgery, Hainan General Hospital, Haikou, Hainan 570311, P.R. China

Received June 17, 2015; Accepted July 20, 2015

DOI: 10.3892/or.2015.4203

Abstract. ADAM15 is a membrane-associated proteinase belonging to a disintegrin and metalloproteinase (ADAM) family. Recent studies suggested that ADAM15 is overexpressed in several types of cancer and is involved in metastatic tumor progression. However, the function of ADAM15 in non-small cell lung cancer (NSCLC) is currently unknown. In the present study, we found that high expression of ADAM15 was associated with decreased overall survival (OS) and disease-free survival (DFS) in NSCLC patients. Furthermore, shRNA-mediated knockdown of ADAM15 attenuated cell migration and invasion. Mechanistic study demonstrated that ADAM15 upregulated MMP9 expression in lung cancer cells via activation of the MEK-ERK pathway. Moreover, ADAM15 proteolytically cleaved and activated pro-MMP9 *in vitro* and interacted with MMP9 *in vivo*. Overexpression of ADAM15 in A549 cells promoted cell invasion, while knocking down MMP9 attenuated cell invasive ability. Therefore, our data not only support a pro-metastatic role of ADAM15 in lung cancer progression, but also reveal a novel mechanism of ADAM15 in promoting cancer cell invasion through directly targeting MMP9 activation.

Introduction

The a disintegrin and metalloproteinase (ADAM) belong to a proteinase family consisting of 40 putative membrane-bound cell surface glycoproteins (1). These zinc-dependent proteases contain an amino terminal metalloproteinase and a disintegrin domain, a cysteine-rich and an EGF-like sequence, a transmembrane and a cytoplasmic domain (2). It has been proven that 13 members of ADAM family are catalytically active through their metalloproteinase domain. These catalytic members play an important role in mediating extracellular matrix protein degradation and shedding of growth factors, cytokines and adhesion molecules. Besides, the disintegrin domain of ADAMs is also involved in binding to integrins to mediate cell-cell and cell-matrix interactions (3). ADAMs have been shown to play important roles in cancer progression (4). ADAM9, ADAM10 and ADAM17 are suggested to cleave epidermal growth factor receptor (EGFR) ligands and HB-EGF to activate EGFR signaling pathway in tumor cells (5-7). Furthermore, ADAMs can cleave many cell adhesion molecules to mediate tumor metastasis. For example, it has been reported that cell adhesion molecule cadherins are substrates for ADAM10 and ADAM15 (8-10). The cleavage of E-cadherin and N-cadherin in tumor cells supports cancer cell migration, invasion and metastasis (11).

As a catalytic active member of ADAM family, ADAM15 is the only ADAM containing an arginine-glycine-aspartic acid (RGD) motif in the disintegrin domain (12). The RGD motif of ADAM15 interacts with integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ to regulate cell adhesion and motility (13). ADAM15 is widely expressed in normal tissues and cancer cell lines. Overexpression of ADAM15 has been reported in many solid tumors including breast, lung, colorectal, ovarian and prostate cancer (14-16). Dependent on the tumor type, ADAM15 either promotes or suppresses cancer progression. For example, in prostate cancer, Najy *et al* demonstrated that ADAM15 promoted cancer cell migration and metastatic progression (17). While Toquet *et al* reported that downregulation of ADAM15 promoted colon cancer metastasis and was corrected with poor prognosis of colon cancer patients (18).

Lung cancer is the most common cancer worldwide and is also the leading cause of malignancy-related diseases worldwide (19). Approximatly 85-90% of lung cancer cases are non-small cell lung cancer (NSCLC) (20). In China, the incidence of lung cancer has constantly increased each year and the mortality rate has markedly increased by 465% over

Correspondence to: Dr Gao Li, Department of Thoracic Surgery, Hainan General Hospital, Haikou, Hainan Province, P.R. China
E-mail: drligao117@163.com

Abbreviations: ADAM, A disintegrin and metalloproteinase; MMP, matrix metalloproteinase; NSCLC, non-small cell lung cancer; DFS, disease-free survival; OS, overall survival; shRNA, short hairpin RNA

Key words: non-small cell lung cancer, A disintegrin and metalloproteinase, matrix metalloproteinase 9, metastasis, cell migration

the past 30 years (21). Although ADAM15 has been reported to be overexpressed in lung carcinomas (22), the prognostic value of ADAM15 in lung cancer is not yet clear, and its function in lung cancer is still unknown. In the present study, we investigated the prognostic value of ADAM15 in Chinese NSCLC cancer patients and studied the roles of ADAM15 in lung cancer cell migration and invasion.

Materials and methods

Reagents. Antibodies to ADAM15 (EPR5619), EGFR, phospho-EGFR, ERK1/2, phospho-ERK1/2, AKT and phosphor-AKT (S473) were purchased from Abcam (Cambridge, MA, USA). Antibodies to β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Matrigel was from Life Technologies (Thermo Fisher Scientific, New York, NY, USA). Recombinant MMP9 was purchased from R&D Systems (Minneapolis, MN, USA). The MMP activity assay kit (cat no. ab112146) was from Abcam.

Patients. A total of 121 lung carcinoma samples were collected at the Hainan General Hospital from January 2004 to December 2008. Before specimen collection, none of the patients had received radiotherapy and chemotherapy. All of the patients diagnosed as NSCLC received cytoreductive surgery before cisplatin-based adjuvant chemotherapies. According to the 2009 WHO/IASLC (23), histopathological classification (Table I) was performed by an experienced pathologist. Disease-free survival (DFS) and overall survival (OS) were analyzed as previously described (24). The present study was approved by the Research Ethics Committee of Hainan General Hospital, Hainan, China. Informed consent was obtained from all the patients.

Immunohistochemistry. Serial paraffin sections were incubated with anti-ADAM15 antibody (1:100 dilution in 5% BSA in PBS) at 4˚C overnight. Rabbit IgG was used as a negative control. After washing three times with PBS, the slides were incubated with biotinylated secondary antibody (1:200 dilution) at room temperature for 30 min. After washing three times with PBS, the slides were stained with the Elite ABC kit (Vector Labs, USA). Finally, the slides were counterstained with hematoxylin, dehydrated, cleared and then mounted with Permount mounting medium (Fisher Scientific, USA). Histological images were captured from the microscope (Carl Zeiss, AX10; Germany) with an objective magnification of x20. ADAM15 staining was independently scored by two pathologists and was calculated using a previously defined scoring system (24). Briefly, the proportion of the positive tumor cell was scored as: 0, <5%; 1+, 5-20%; 2+, 21-50%; 3+, 50-70% and 4+, 70-100%. The intensity was arbitrarily scored as 0, weak (no color or light blue); 1, moderate (light yellow); 2, strong (yellow brown) and 3, very strong (brown). The overall score was calculated by multiplying the two scores obtained from each sample. A score of ≥8 was defined as a high ADAM15 expression and scores of <8 defined as a low ADAM15 expression.

Cell lines. The human lung cancer cell lines A549, NCI-H1648 and NCI-H647 were purchased from ATCC and cultured at 37˚C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin G, 100 μg/ml streptomycin and 2 mM L-glutamine.

Table I. Primers used in real-time PCR assay.

Oligonucleotide		Sequence (5'→3')	Purpose
Human MMP1	F	ctggccacaactgccaaatg	RT-PCR
Human MMP1	R	ctgtccctgaacagcccagtactta	RT-PCR
Human MMP2	F	tctcctgacattgaccttggc	RT-PCR
Human MMP2	R	caaggtgctggctgagtagatc	RT-PCR
Human MMP3	F	attccatggagccaggctttc	RT-PCR
Human MMP3	R	catttgggtcaaactccaactgtg	RT-PCR
Human MMP7	F	tgagctacagtgggaacagg	RT-PCR
Human MMP7	R	tcatcgaagtgagcatctcc	RT-PCR
Human MMP9	F	ttgacagcgacaagaagtgg	RT-PCR
Human MMP9	R	gccattcacgtcgtccttat	RT-PCR
Human MMP10	F	gtcacttcagctcctttcct	RT-PCR
Human MMP10	R	atcttgcgaaaggcggaact	RT-PCR
Human β-actin	F	tccctggagaagagctacg	RT-PCR
Human β-actin	R	gtagtttcgtggatgccaca	RT-PCR

F, forward; R, reverse.

Generating shRNA or siRNA knockdown cells. The lentivirus silencing vector expressing shRNA targeting human ADAM15 (TRC no. TRCN0000371222) was obtained from Sigma-Aldrich (St. Louis, MO, USA). This silencing and the empty vectors alone (TRC2-pLKO-puro, as control) along with pxPAX2 and pCMV-VSVG were transfected into 293T cells to produce lentivirus. After 24 h of transfection, supernatant was collected and this lentiviral preparation was used to infect cells. Stably infected cells were isolated in selection media containing puromycin (2 μg/ml).

For siRNA treatments, A549 cells were plated in a 3.5 cm dish and transfected with 50 pm MMP9 or non-targeting siRNA (Human On-TARGETplus siRNA pools of four oligos; Dharmacon, USA) using Lipofectamine RNAi Max (Invitrogen). After transfection 48 h, cells were used for invasion assay.

Plasmid and transfection. ADAM15 full-length sequence (NM_207191) was amplified from reverse transcripted cDNA from A549 cells. The ADAM15 sequence was cloned into the mammalian expression vector pCMV-3Tag-3 (Agilent Technologies, USA). DNA sequencing confirmed the sequence fidelity. Then, A549 cells were transfected with pCMV-3Tag-3-ADAM15 plasmid or empty control vector using Lipofectamine 2000 (Invitrogen). After 24 h, cells were collected and used for the experiment.

Wound-healing migration assay. Cells were starved in DMEM containing 0.5% FBS for 6 h to inactivate cell proliferation and then wounded by pipette tips (25). The final concentration

of 10% FBS was added into the culture medium to stimulate cell migration. After 8 h of migration, cells were labeled with 2 mg/ml calcein-AM (Invitrogen) for 30 min and visualized using an inverted microscope (magnification, x10; Olympus, Tokyo, Japan). Migrated cells were manually quantified. Three independent experiments were performed.

Transwell migration assay. A Transwell migration assay with 6.5-mm-diameter polycarbonate filters (8-μm pore size) was used. The filter of the Transwell plate (BD Biosciences) was coated with Matrigel. The bottom chambers were filled with 500 μl of DMEM containing 10% FBS. Cells ($4x10^4$) suspended in 100 μl of DMEM containing 0.5% FBS were seeded in the top chambers. Cells were allowed to migrate for 24 h. Non-migrated cells were removed with cotton swabs, and migrated cells were fixed with cold 4% paraformaldehyde and stained with 1% crystal violet. Images were captured using an inverted microscope (magnification, x10; Olympus), and migrated cells were quantified by manual counting.

Western blotting and immunoprecipitation. Cells were lysed in M2 lysis buffer [150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 1% Nonidet P-40] containing a protease inhibitor mixture (Roche Applied Science) and a phosphatase inhibitor mixture (St. Louis, MO, USA). The equal amount of total protein was subjected to SDS-PAGE analysis and immunoblotting with the appropriate antibodies.

For immunoprecipitation, lysates were precipitated with antibody and protein G-agarose beads by incubation at 4˚C overnight. Beads were washed four times with 1 ml M2 buffer, and the bound proteins were removed by boiling in SDS buffer and resolved in 4-20% SDS-polyacrylamide gels for western blot analysis.

Real-time PCR. TRIzol reagent (Invitrogen) was used to isolate total RNA. The cDNA was prepared using reverse transcriptase SuperScript II (Invitrogen) with 2 μg of DNase I-treated total RNA. Then, 2 μl of the cDNA was mixed with an 18 μl PCR assay mixture containing 0.5 M each primer and 1 μl Brilliant SYBR®-Green QPCR Master Mix (Stratagene, USA). PCR was conducted with the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, USA) using the following conditions: 95˚C for 10 min followed by 40 cycles of 95˚C for 15 sec, 55˚C for 30 sec and 72˚C for 30 sec. The primers are listed in Table I. The threshold cycle number for each MMP was normalized to that of β-actin, and the resulting value was converted to a linear scale. All assays were performed at least three times from independent RNA preparations.

MMP activity assay. The MMP activity was determined using an assay kit from Abcam according to the manufacturer's protocol. Briefly, $5x10^5$ cells were seeded into 6-well plates and allowed to attach overnight, then starved with serum-free media for another 12 h. Cells were then stimulated with 10% FBS and 50 μl of medium was removed at different time points. The conditioned medium was mixed with 50 μl of 2 mM APMA working solution and incubated for 15 min at 20˚C followed by addition of 100 μl of the fluorogenic peptide substrate solution in the kit. A microplate reader with a filter set of Ex/Em = 490/525 nm was used to measure the fluorescence activity.

Table II. Clinicopathological characteristics and results of ADAM15 immunohistochemistry.

Characteristics	No. of patients	ADAM15 expression		P-value
		Low/no	High	
Age (years)				0.7161
≤60	54	24	30	
>60	67	32	35	
Gender				0.7552
Male	76	36	40	
Female	45	20	25	
Histological type				0.8626
Squamous cell carcinoma	68	31	37	
Adenocarcinoma	53	25	28	
Lymph node metastasis				0.0003
Negative	48	32	16	
Positive	73	24	49	
Differentiation				0.6142
Well	64	31	33	
Moderate/poor	57	25	32	
TNM stage				0.7209
I	25	13	12	
II	42	20	22	
III	54	23	31	

ADAM, a disintegrin and metalloproteinase.

Gelatin zymography. The cell culture supernatants were collected and mixed with non-reducing SDS gel sample buffer and was loaded to a 10% polyacrylamide gel containing 0.1% SDS and 1 mg/ml gelatin. After electrophoresis, the gels were incubated with renaturing buffer containing 50 mmol/l Tris-HCl (pH 7.5), 0.15 mol/l NaCl, 5 mmol/l $CaC1_2$, 5 μmol/l ZnCl, 0.02% NaN_3, 0.25% Triton X-100 at room temperature for 30 min, and then were incubated in the same buffer without Triton X-100 at 37˚C overnight. Then, the gels were stained by Coomassie brilliant blue R-250 solution.

Statistical analysis. The relationship between the expression of ADAM15 and the patients clinical characteristics were analyzed using the χ^2 or Fisher's exact tests, as appropriate. OS and DFS curves were generated using Kaplan-Meier method and compared using the log-rank test. Univariate and multivariate analyses were performed using Cox regression models. P-value <0.05 was regarded as statistically significant. Data are analyzed using SPSS (version 20.0; IBM Corporation, Armonk, NY, USA) software program. Results are expressed as the means ± SD and are representative of at least three separate experiments. The two-sample t-test was

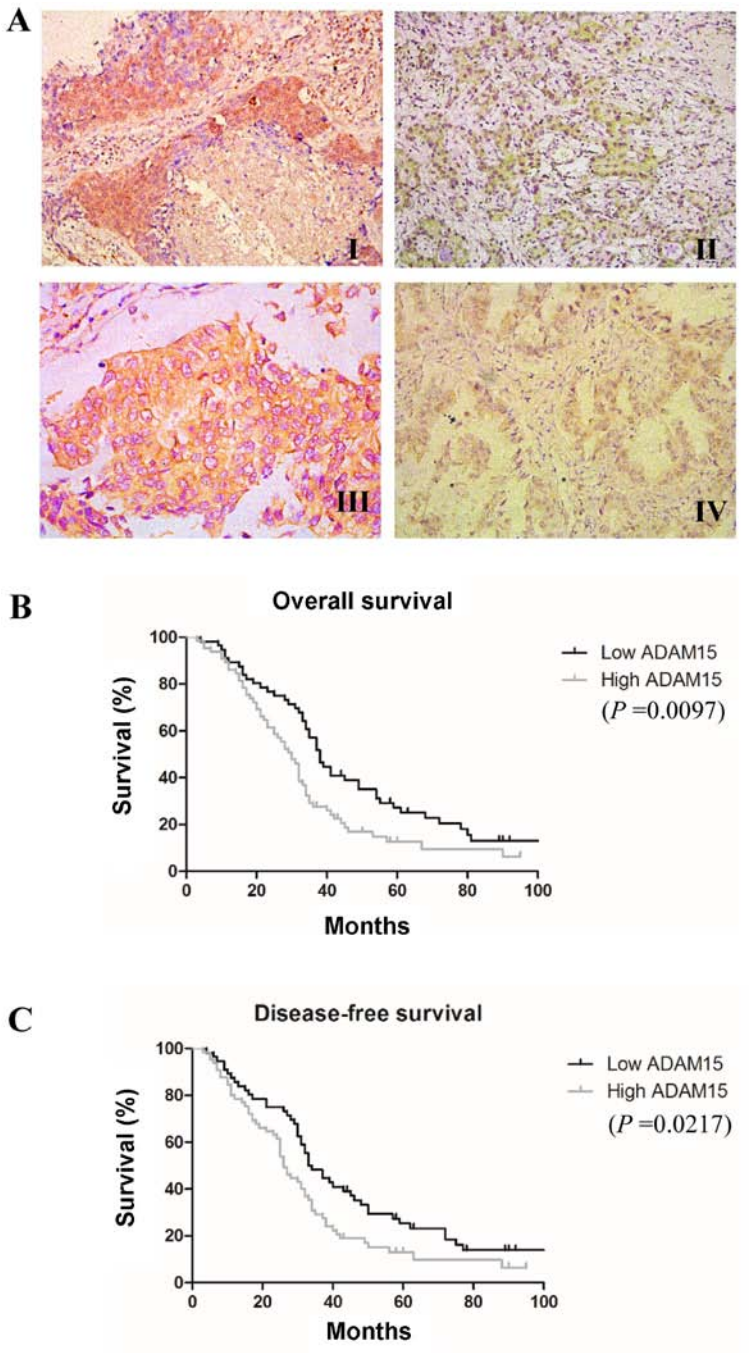

Figure 1. ADAM15 correlates with decreased overall survival (OS) and disease-free survival (DFS) in NSCLC patients. (A) Representative images of ADAM15 immunohistochemical staining in NSCLC carcinomas. I, High and II, low ADAM15 expression in squamous cell carcinoma; III, high and IV, low ADAM15 expression in adenocarcinoma. Magnification, x200. (B) Kaplan-Meier log-rank survival analyses for overall survival and (C) disease-free survival of NSCLC patients according to ADAM15 expression. NSCLC, non-small cell lung cancer; ADAM, a disintegrin and metalloproteinase.

used to determine statistical differences of the means of two groups.

Results

High expression of ADAM15 is associated with poor prognosis in NSCLC patients. To investigate the role of ADAM15 in lung cancer, we first examined the expression of ADAM15 in NSCLC carcinomas. The tissue samples were collected from 121 NSCLC patients with median age of 63 years. Patient characteristics of the population are summarized in Table II. At the time of last follow-up, 85% of patients had died and 8.2% had no evidence of disease. As shown in Fig. 1A, ADAM15-positive staining was localized to the cytoplasm as well as on the cell surface in tumor cells, which is similar to a previous study (22). We further analyzed the association of ADAM15 expression with clinicopathological characteristics of the patients. We found that ADAM15 expression was significantly associated with lymph node metastasis (Table II). However, no statistical correlations were observed between ADAM15 expression and the patients age, gender, histologic types, tumor differentiation stages or TNM stages (Table II).

Table III. Mutivariate analyses for all the patients (n=121).

Characteristics	DFS		OS	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Age (years) ≤60 vs. >60	1.6 (0.7-2.0)	0.2551	1.5 (0.7-1.7)	0.5431
Gender Male vs. female	1.3 (0.6-1.5)	0.4124	0.9 (0.4-1.8)	0.2356
Histological type Squamous vs. adenocarcinoma	1.4 (0.7-1.5)	0.2097	1.3 (0.5-1.5)	0.1093
Lymph node metastasis Positive vs. negative	3.9 (0.9-5.0)	0.0016	4.0 (0.8-6.4)	0.0024
Differentiation Well vs. moderate to poor	1.0 (0.3-1.2)	0.2632	1.5 (0.7-1.8)	0.1736
TNM stage I vs. II vs. III	0.8 (0.6-1.8)	0.4862	0.9 (0.5-1.2)	0.2156
Level of ADAM15 expression High vs. low	4.1 (0.7-7.3)	0.0042	6.1 (1.8-9.5)	0.0233

HR, hazard ratio; CI, confidence interval; DFS, disease-free survival; OS, overall survival; ADAM, a disintegrin and metalloproteinase.

We then evaluated the prognostic significance of ADAM15 expression in NSCLC patients. We found that high ADAM15 expression was significantly associated with decreased OS (median 30 vs. 38 months, P=0.0097) and DFS (median 26 vs. 33 months, P=0.0217) (Fig. 1B and C). Furthermore, a multivariate Cox regression analysis was applied to all of the clinicopathological characteristics with ADAM15 expression levels. As shown in Table III, high expression of ADAM15 were independently associated with poor prognosis in NSCLC patients.

Knockdown of ADAM15 in lung cancer cells attenuates cell migration and invasion. To further investigate the function of ADAM15 in lung cancer progression, we used a shRNA-mediated approach to suppress the expression of ADAM15 in lung cancer cells. As shown in Fig. 2A, the shRNA specific for ADAM15 efficiently eliminated its expression in multiple metastatic lung cancer cell lines including A549, NCI-H1648 and NCI-H647 cells. Since we found that ADAM15 overexpression is correlated with lymph node metastasis, we then asked whether ADAM15 downregulation influence cell migration and invasion. To measure the cell migration ability, a wound-healing migration assay was performed in both vector control and shRNA-ADAM15 A549 cells. Notably, we found that knockdown of ADAM15 significant delayed wound closure rate in A549 cells (Fig. 2B). Then, a Matrigel Transwell assay was performed to evaluate the cell invasion ability. As shown in Fig. 2C, knockdown of ADAM15 attenuated the invasion ability of lung cancer cells. In addition, no change of cell cycle or cell proliferation was observed in shRNA-ADAM15 A549 cells (data not shown). Thus, our data suggest that ADAM15 plays an important role in mediating lung cancer cell migration and invasion.

ADAM15 upregulates MMP activity and the expression of MMP9 in lung cancer cells. Tumor cell migration and metastasis are controlled by the interactions between surface adhesion molecules and surrounding microenvironment. In this scenario, matrix metalloproteinases (MMPs) play a critical role in tumor invasion and metastasis by modulating cell-cell and cell-extracellular matrix (ECM) interactions. We then asked whether shRNA-mediated knockdown of ADAM15 influences MMP activity in lung cancer cells. Notably, we found that FBS-induced MMP activation was decreased in ADAM15 knockdown cells compared to shRNA control cells (Fig. 3A). Moreover, overexpression of ADAM15 in A549 cells significantly increased MMP activity, further suggesting that ADAM15 upregulates MMP activity in lung cancer cells (Fig. 3B and C). To determine whether upregulation of MMP expression contribute to increased MMP activity in ADAM15 overexpressed cells, we examined expression of several MMP members in both control and ADAM15 overexpressed cells by real-time PCR. Notably, we found that only MMP9 expression was significantly increased in ADAM15 overexpressed cells (Fig. 3D). Moreover, knockdown of ADAM15 downregulated MMP9 expression in A549 cells, suggesting ADAM15 upregulated MMP9 expression in lung cancer cells (Fig. 3E). It was reported that the MEK-ERK and PI3K-Akt pathways upregulated MMP9 expression in cancer cells (26,27). We examined the activation of these pathways in ADAM15 overexpressed cells. As shown in Fig. 3F, overexpression of ADAM15 in A549 cells increased phosphorylation levels of EGFR and ERK, while phosphorylation of Akt was not changed in ADAM15 overexpressed cells. We then treated cells with an ERK inhibitor PD98059 and we found that PD98059 significantly attenuated ADAM15-induced MMP9 expression (Fig. 3G). Thus, these data suggested that ADAM15

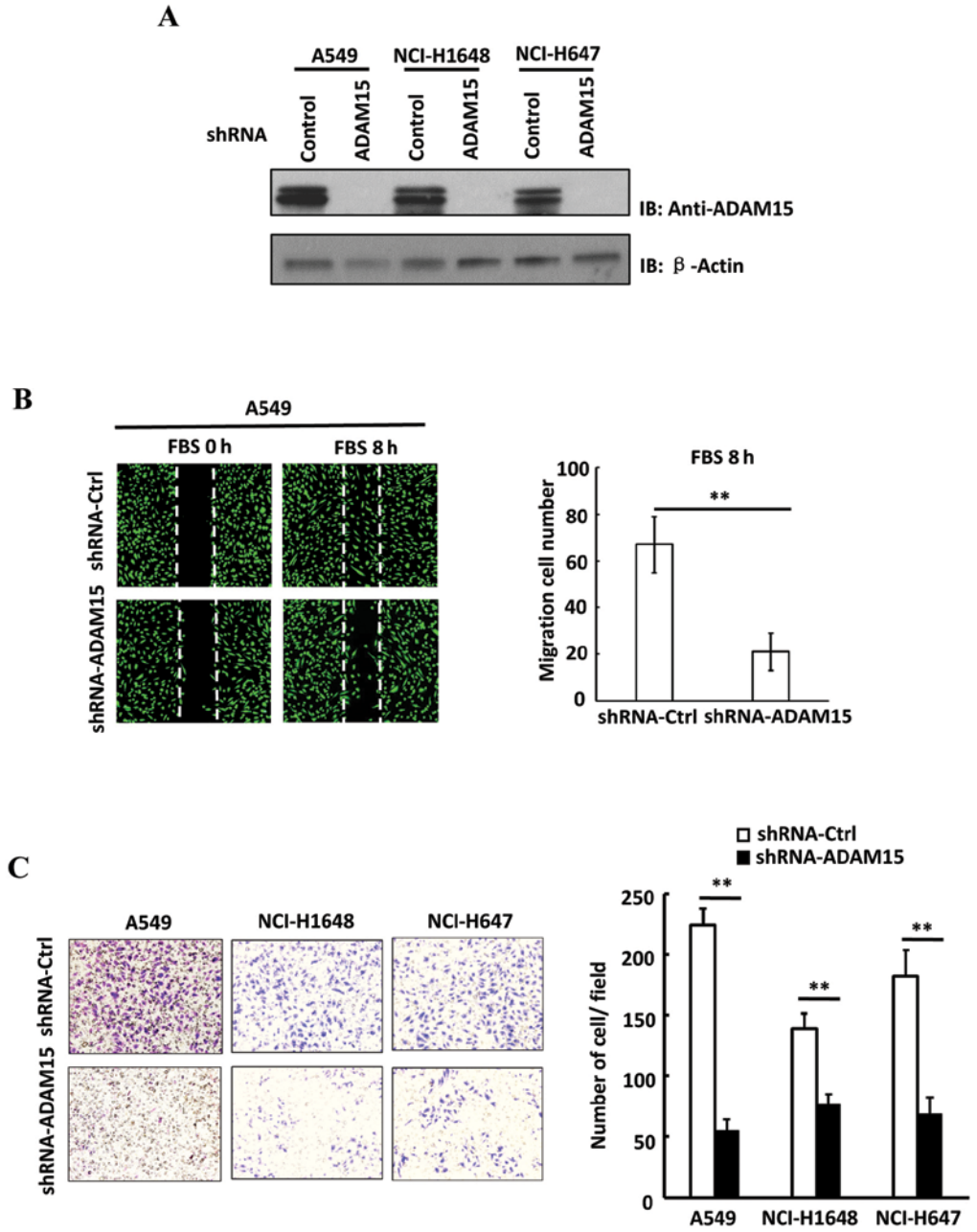

Figure 2. Knockdown of ADAM15 in lung cancer cells inhibits cell migration and invasion. (A) Expression of ADAM15 in control or ADAM15 shRNA knockdown cells. (B) Control or ADAM15 shRNA A549 cells were scratched by pipette and stimulated with 10% FBS for 8 h. Left, representative images of wound-healing. Right, quantitative data of migrated cells. (C) Control or ADAM15 shRNA cells were seeded in Transwells covered with Matrigel and cultured for 24 h. Left, representative images of Transwell assay. Right, quantitative data of invasive cells. **P<0.01; relative to untreated cells. Data are representative results of three independent experiments. ADAM, a disintegrin and metalloproteinase; FBS, fetal bovine serum.

upregulated expression of MMP9 via MEK-ERK pathway in lung cancer cells.

ADAM15 activates and interacts with MMP9 in lung cancer cells. To study ADAM15-dependent MMP9 activity in lung cancer cells, we used gelatin zymography to measure the activity of MMP9 in ADAM15-shRNA knockdown cells. We found that FBS-induced MMP9 activation was decreased in ADAM15 knockdown cells (Fig. 4A). We used purified enzymes to examine the role of ADAM15 in MMP9 activation. Notably, we found that incubation of ADAM15 and pro-MMP9 converts pro-MMP9 to lower molecular weight species corresponding to its active form, suggesting that ADAM15 proteolytically cleave and activate pro-MMP9 *in vitro* (Fig. 4B). Furthermore, we found that ADAM15 interacted with MMP9 in A549 cells as examined by co-immunoprecipitation (Fig. 4C). Taken together, our data demonstrated that ADAM15 interacts with MMP9 and promotes maturation and activation of pro-MMP9 through a proteolytic mechanism.

MMP9 activity is required for ADAM15-regulated cell invasion. To study the role of MMP9 in ADAM15-regulated cell invasion, we used a small interfering RNA targeting MMP9 to silence MMP9 in ADAM15-overexpressed A549 cells and analyzed its invasive ability (Fig. 4A). As shown in Fig. 4B,

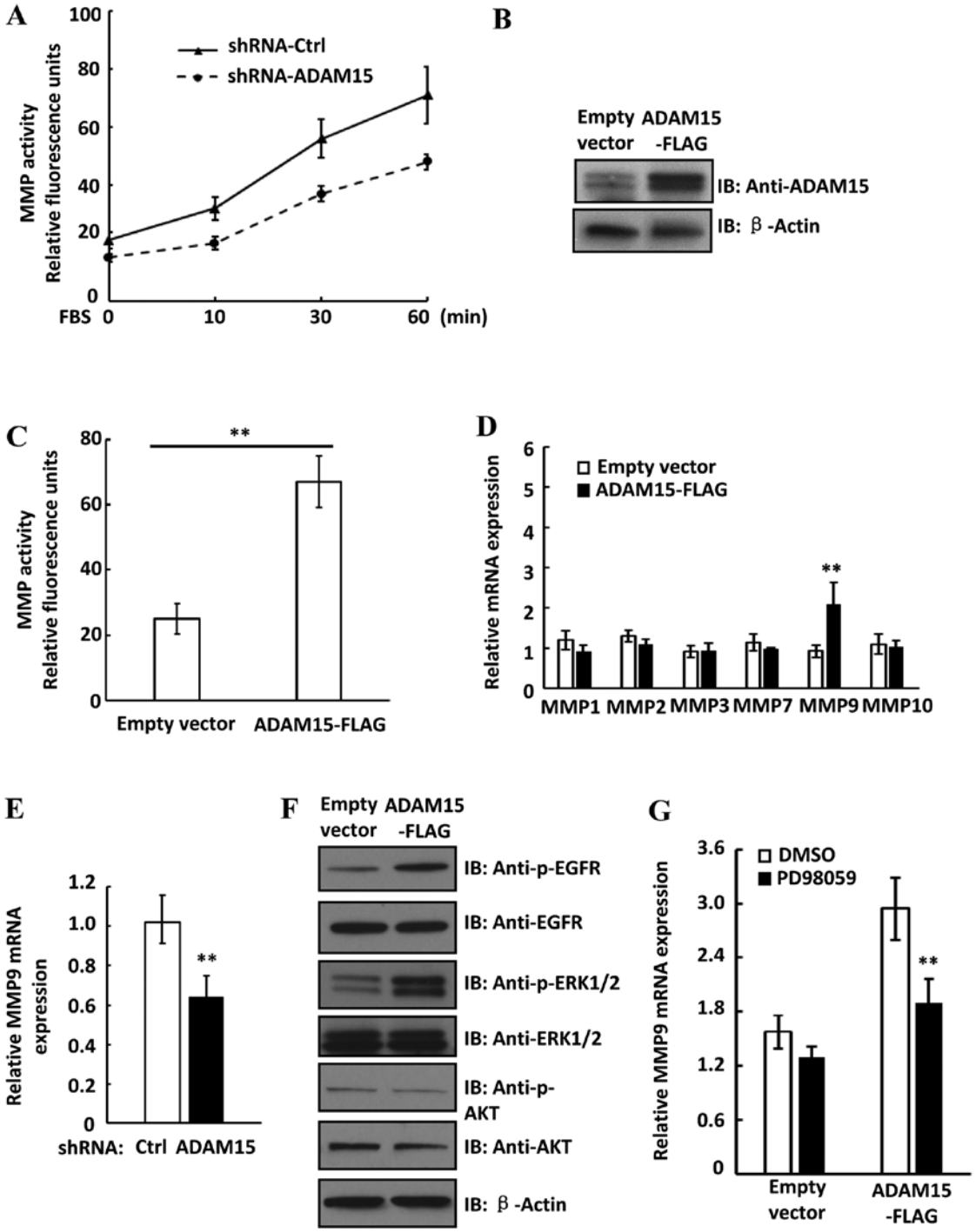

Figure 3. ADAM15 increases MMP activity and expression of MMP9 in lung cancer cells. (A) Control or ADAM15 shRNA A549 cells were serum starved for 12 h and then stimulated with 10% FBS. The metalloproteinase activities at different time points were determined using a fluorogenic substrate as described in Materials and methods. (B) Control vector or ADAM15-FLAG vector were transfected into A549 cells and the expression of ADAM15 was analyzed by western blotting. (C) The metalloproteinase activities were analyzed in both control and ADAM15 overexpressed A549 cells. (D) The mRNA expression levels of MMP family members were analyzed by real-time PCR in both control and ADAM15 overexpressed A549 cells. (E) The expression of MMP9 in both control and ADAM15 shRNA A549 cells was analyzed by real-time PCR. (F) Total cell lysates from control and ADAM15 overexpressed A549 cells were subjected to western blot analysis and specific antibodies involved in EGFR signaling pathway as indicated. (G) Control or ADAM15 overexpressed A549 cells were treated with MAPK inhibitor PD98059 or DMSO control for 12 h and the expression of MMP9 was analyzed by real-time PCR. $^{**}P<0.01$; relative to control cells. Data are representative results of three independent experiments. ADAM, a disintegrin and metalloproteinase; FBS, fetal bovine serum; DMSO, dimethylsulfoxide.

overexpression of ADAM15 promoted cell invasion, while silencing MMP9 decreased invasiveness. Moreover, both ERK inhibitor PD98059 and MMP9 inhibitor I attenuated cell invasion in A549-ADAM15 cells (Fig. 4C). Conversely, overexpression of MMP9 in ADAM15 knockdown cells promoted cell invasion (Fig. 4D). Collectively, our data demonstrated that ADAM15-induced MMP9 activation, at least in part, was involved in ADAM15-regulated lung cancer cell invasion.

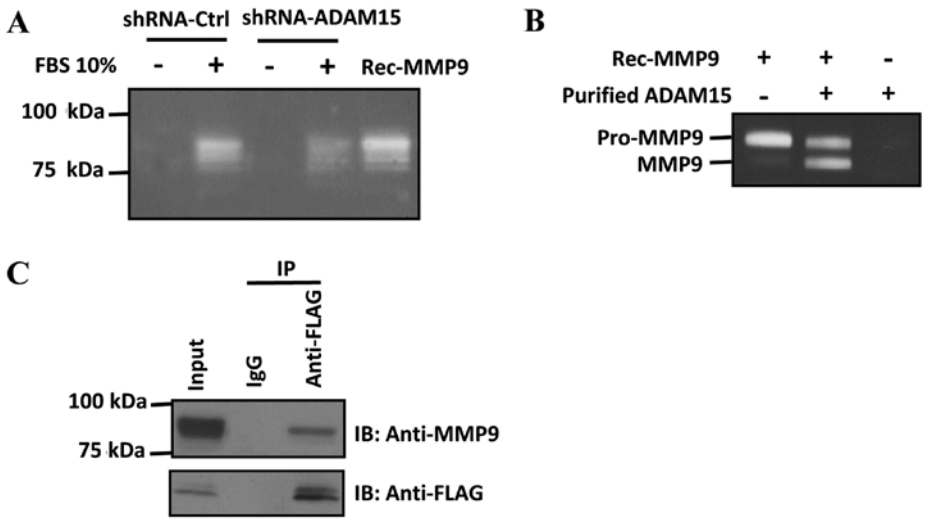

Figure 4. ADAM15 activates MMP9 and interacts with MMP9 in A549 cells. (A) Control or ADAM15 shRNA A549 cells were serum starved for 12 h and then were stimulated with 10% FBS for 8 h. The conditioned medium was analyzed for MMP9 activity using a gelatin zymography. Recombinant MMP9 was used as a positive control. (B) Recombinant MMP9 (50 nM) and purified ADAM15 by immunoprecipitation from $2x10^6$ A549 cells were incubated at 37˚C for 16 h in collagenase buffer and analyzed by gelatin zymograhpy. (C) A549 cells were transfected with ADAM15-FLAG vector. After 24 h, cell lysates were immunoprecipitated with anti-FLAG antibody (IP:FLAG) and analyzed by immunoblotting with anti-MMP9 or anti-FLAG antibodies. ADAM, a disintegrin and metalloproteinase; FBS, fetal bovine serum.

Discussion

It has been reported that ADAM15 was overexpressed in lung carcinomas (22). However, the prognosis value of ADAM15 in lung carcinomas remains elusive. In the present study, we found that ADAM15 represents an independent prognostic factor for the outcome in Chinese NSCLC patients. Using a relatively large cohort of lung carcinoma specimens, we observed that high expression of ADAM15 correlated with decreased disease-free survival (DFS) and overall survival (OS) in lung cancer patients. Increased expression of ADAM15 has been reported in several types of cancer including ovary, gastric, breast and prostatic cancers. In breast and prostatic cancers, overexpression of ADAM15 was highly related to metastatic cancer progression, suggesting a pro-metastatic role of ADAM15 (14). However, in colon cancer, the decreased expression of ADAM15 was associated with cancer metastasis and poor prognosis of colon cancer patients (18). Thus, these results reveal that ADAM15 plays different roles in the context of various types of cancer.

Although ADAM15 has been shown to be overexpressed in lung carcinomas, the role of ADAM15 in lung cancer progression is currently unknown. We found that knockdown of ADAM15 attenuated migratory and invasive capacity in multiple lung cancer cell lines, suggesting a pro-metastatic role of ADAM15 in lung cancer. Notably, a similar observation was also reported in prostate cancer cells (17). Matrix metalloproteinase (MMPs) are a family of zinc endopeptidases that degrade extracellular matrix components in either normal physiological conditions or carcinogenesis (28). Since the degradation of extracelluar matrix is one of the key steps in cancer invasion and metastasis, we measured the MMP activity in ADAM15 knockdown cells. Notably, we found that ADAM15 increased MMP activity in A549 cells. Furthermore, we observed that ADAM15 activated EGFR-MEK-ERK pathway and thus upregulated MMP9 expression. As a plasma membrane-associated proteinase, ADAM15 sheds a variety of cell surface molecules including cytokines and adhesion molecules. It has been reported that ADAM15 cleaved E-cadherin to generate a soluble fragment and in turn simulates EGFR signaling pathway in breast cancer cells (9). We also observed that overexpression of ADAM15 promoted extracellular shedding of E-cadherin in A549 cells (data not shown). Thus, the activation of EGFR-MEK-ERK signaling pathway by ADAM15 in lung cancer cells could be due to the increased shedding of cell surface molecules such as E-cadherin. Future studies need to address whether the soluble E-cadherin or other cell surface molecules contribute to EGFR pathway activation by ADAM15 in lung cancer cells.

MMP9 is first synthesized as a proenzyme (pro-MMP9) and requires cleavage by other proteinases to achieve its catalytic activity. For example, it has been shown that pro-MMP9 was proteolytically processed and activated by plasmin. Other MMPs, including MMP-2, MMP-3 and MT1-MMP, were also involved in the activation of MMP9. We have found that ADAM15 upregulated MMP9 expression in lung cancer cells. Since regulation of MMP activities can be achieved at multiple levels, our data further demonstrated that ADAM15 interacted with MMP9 and proteolytically cleaved and activated pro-MMP9 *in vitro*. Other members of the ADAM family have been reported to regulate MMP activities in cancer cells. For example, ADAM12 together with $\alpha_v\beta_3$ integrin form a ternary protein complex with MMP-14 to activate MMP-14 in breast cancer cells (29). ADAM17 upregulates MMP-2 and MMP-9 expression to promote prostate cancer cell invasion (30). Thus, our study identified a novel ADAM15-mediated activation mechanism of MMP-9 in lung cancer cells.

As an important matrix proteinase, MMP9 degrades collagen type IV on the basement membrane. Overexpression

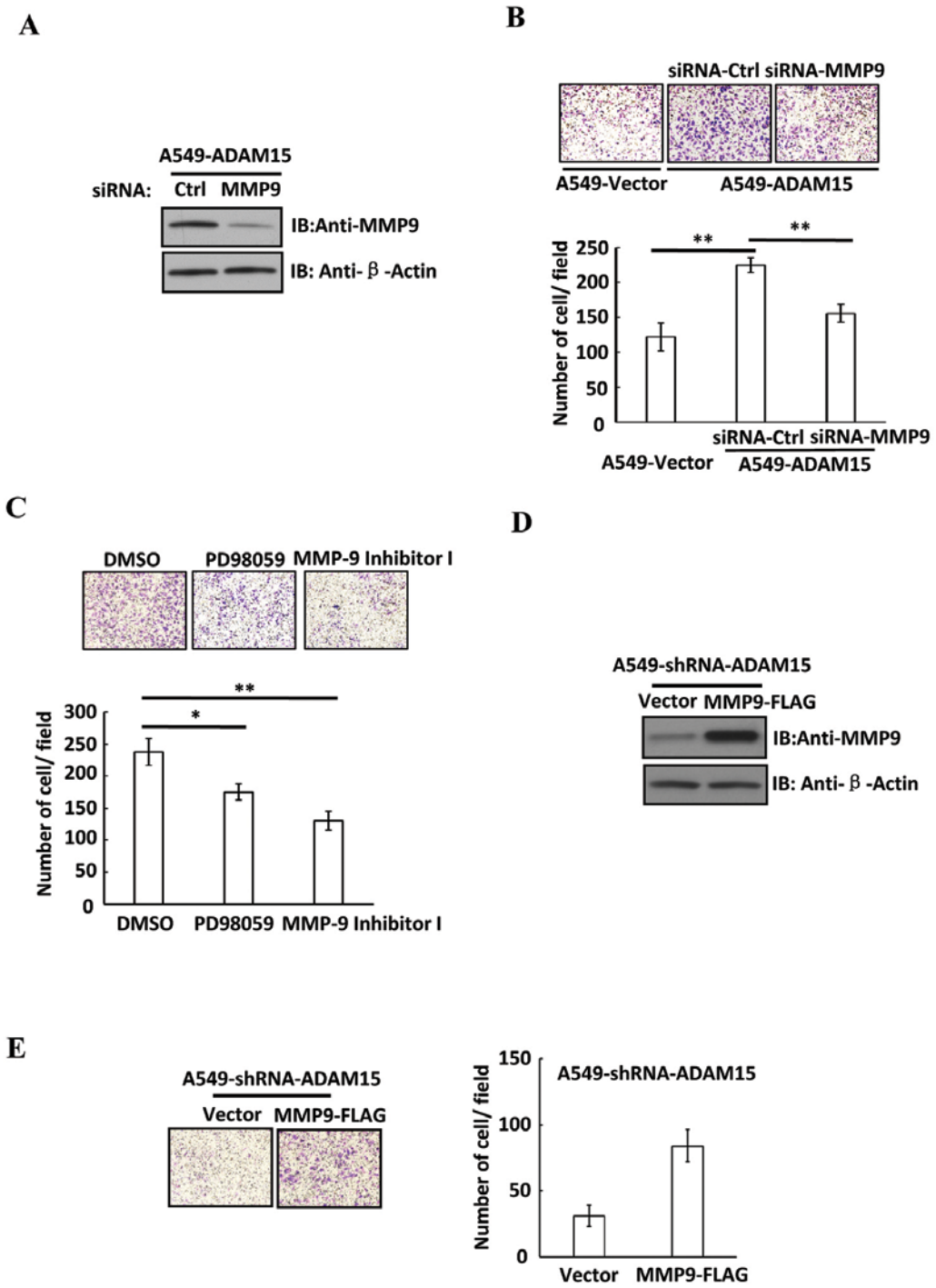

Figure 5. MMP9 activity is required for ADAM15-regulated cell invasion. (A) Knockdown of MMP9 by siRNAs in ADAM15 overexpressed cells as shown by western blotting. (B) Control, ADAM15 overexpressed cells or MMP9 siRNA cells were subjected to Matrigel invasion assays. Upper, representative images of Transwell assay. Lower, quantitative data of invasive cells. (C) A549 cells were treated with DMSO control, MAPK inhibitor PD98059 or MMP9 inhibitor I and subjected to Matrigel invasion assays. Upper, representative images of Transwell assay. Lower, quantitative data of invasive cells. (D) Overexpression of MMP9 in ADAM15 shRNA A549 cells as shown by western blotting. (E) Control or ADAM15 overexpressed cells were subjected to Matrigel invasion assays. Left, representative images of Transwell assay. Right, quantitative data of invasive cells. **P<0.01; relative to control cells. Data are representative results of three independent experiments. ADAM, a disintegrin and metalloproteinase.

of MMP9 has been reported in different types of cancer and it is believed to facilitate tumor invasion and metastasis (28,31). It has been reported that EGFR signaling pathway upregulated MMP9 expression in several types of cancer, including NSCLC (26,32,33). In NSCLC patients, MMP9 expression was strongly correlated with EGFR expression and the co-expression of these two proteins was associated with poor outcome in the patients (26). Our data showed that MMP9 was involved in ADAM15-regulated cancer cell invasion. Knockdown of MMP9 prevented ADAM15-induced cell invasion, while overexpression of MMP9 promoted cell invasion in ADAM15 knockdown cells. These results may suggest an important functional link among ADAM15, EGFR signaling pathway and MMP9 in the metastasis

and progression of NSCLC. In future studies, it would be interesting to investigate the association among ADAM15, EGFR and MMP9 expression in NSCLC patients and re-evaluate their prognostic values.

In conclusion, out study explored the expression of ADAM15 in NSCLC and suggested that high expression of ADAM15 correlates with poor outcome in NSCLC patients. We further demonstrated that ADAM15 directly activates MMP9 to promote cell migration and invasion in lung cancer cells. Taken together, the present study suggests that ADAM15 serves as a potential therapeutic target in NSCLC patients.

Acknowledgements

The present study was supported by funding from the Natural Science Foundation of Hunan Province, China (grant no. 2015JJ6058), and the Science and Technology Program of Health and Family Planning Commission of Hunan Province, China (grant no. B2015-111).

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