

# Overexpression of FOXM1 is associated with EMT and is a predictor of poor prognosis in non-small cell lung cancer

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**Abstract.** Forkhead box M1 (FOXM1), a member of the Fox family of transcriptional factors, is considered to be an independent predictor of poor survival in many solid cancers. However, the underlying mechanism is not yet clear. The aim of the present study was to investigate the clinical significance of the correlation between FOXM1 and epithelial-mesenchymal transition (EMT) in non-small cell lung carcinoma and the possible mechanism responsible for FOXM1-induced EMT and metastasis. In the present study, expression levels of FOXM1 and EMT indicator proteins were determined by tissue microarray (TMA) and immunohistochemical staining, western blotting and reverse transcription-PCR (RT-PCR). Other cellular and molecular approaches including gene transfection, small interfering RNA (siRNA), and migration and invasion assays were utilized. Our results demonstrated that FOXM1 overexpression was statistically significantly associated with a higher TNM stage ( $p=0.036$ ), lymph node metastasis ( $p=0.009$ ) and a positive smoking history of the patients ( $p=0.044$ ). Additionally, high expression of FOXM1 correlated with loss of E-cadherin expression ( $p<0.001$ ) and anomalous immunopositivity of Vimentin ( $p=0.002$ ). Moreover, patient survival analysis demonstrated that high expression of FOXM1 ( $p=0.043$ ) and the presence of lymph node metastasis ( $p=0.042$ ) were independent prognostic factors for non-small cell lung cancer (NSCLC). Furthermore, various *in vitro* experiments indicated that overexpression or knockdown of FOXM1 expression altered EMT through activation or inhibition of the AKT/p70<sup>S6K</sup> signaling pathway.

Collectively, the results suggest that FOXM1 may be used as a prognostic indicator for patients with NSCLC and promotes metastasis by inducing EMT of lung cancer cells through activation of the AKT/p70<sup>S6K</sup> pathway. Therefore, we suggest that FOXM1 may be a potential target for lung cancer therapy.

## Introduction

Non-small cell lung cancer (NSCLC) is the leading cause of cancer-related mortality worldwide and is associated with a high rate of metastasis (1). Although metastasis is responsible for as much as 90% of cancer-associated deaths, it remains the most poorly understood aspect of cancer pathogenesis (2).

Forkhead box M1 (FOXM1), a member of the Fox family of transcriptional factors, belongs to a group of evolutionarily conserved transcriptional regulators that are characterized by the presence of a DNA-binding domain called the Forkhead box or the winged helix domain (3,4). Generally speaking, FOXM1 plays a critical role in cell cycle progression by regulating G1/S and G2/M phase transitions of the cell cycle and ensuring the proper execution of mitotic cell division (3-5). It has been reported to be overexpressed in a variety of tumors, including lung, liver, breast and bladder cancer, clear cell renal cell carcinoma and early stage cervical cancer (6-11). Furthermore, increased expression of FOXM1 has been associated with poor prognosis in cancer patients and is considered to be an independent predictor of poor survival in many solid cancers (6,7,10-14). Importantly, downregulation of FOXM1 leads to the inhibition of invasion of pancreatic cancer (15), prostate cancer (16), clear cell renal cell carcinoma (14) and NSCLC (12) cells.

Epithelial-mesenchymal transition (EMT) is an essential phenotypic event during embryonic development, tissue remodeling and wound healing, and plays an essential role in tumor invasion and metastasis (17-19). EMT is also a reversible process that often occurs at the invasive front of many metastatic cancers (20). Loss of E-cadherin and relocalization of  $\beta$ -catenin from the membrane to the nucleus are frequently observed in tumor cells undergoing EMT (17,21). Additionally, expression of the intermediate filament protein Vimentin, which is known to induce changes in cell motility, is a classic

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marker of EMT (22). Although both FOXM1 and EMT play important roles in the metastasis of cancer, the interaction between them remains unclear.

The PI3K/AKT pathway regulates EMT, which is a fundamental event that is thought to predict tissue invasion and metastatic potential (23,24). PI3K and AKT also promote tissue invasiveness and downregulate expression of E-cadherin, a key marker of EMT (25-28). Additionally, Park *et al* found that FOXM1b induced an EMT-like phenotype by activating the AKT-Snail pathway in hepatocellular carcinoma (HCC) (29). However, there is no report on the important role of AKT in FOXM1-induced EMT in NSCLC.

In the present study, we sought to determine whether FOXM1 expression is associated with EMT in NSCLC specimens and whether these factors can predict the clinical outcome and survival of these cancer patients. In addition, we examined whether a strong correlation exists between FOXM1 expression and the EMT process in NSCLC cells and whether the AKT signaling pathway is involved in FOXM1-regulated EMT.

## Materials and methods

**Tissue samples and tissue microarray construction.** The preparation of tissue specimens and the construction of the tissue microarray (TMA) were performed as described previously (30).

**Immunohistochemistry.** Immunohistochemical analysis of TMA slides was carried out as previously reported (30). The following primary monoclonal antibodies were used: FOXM1 rabbit mAb (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), E-cadherin and Vimentin rabbit mAb (1:100; Cell Signaling Technology, Beverly, MA, USA). The intensity score was defined as follows: no signal, 0; weak, 1; moderate, 2 and intense, 3. The score was based on the fraction of immunoreactive cells (0-100%). The total score was calculated by multiplying the intensity score and the fraction score, yielding a total score ranging from 0 to 300%. For statistical analyses, scores of 0-100% were considered low expression, and scores of 101%-300% were considered high expression. Two of the pathologists, without prior knowledge of the clinical data, independently graded the staining intensity in all cases.

**Cell culture, reagents and treatments.** The lung cancer cell lines A549 and H1299 were cultured in Dulbecco's modified Eagle's minimum (DMEM) and H1650, H1975 in RPMI-1640 medium (both from Gibco-BRL), respectively, supplemented with 10% fetal bovine serum (FBS) (HyClone Inc., USA). These cell lines were incubated under standard conditions at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. The PI3K inhibitor LY294002 (10 µmol/l) and the activator epidermal growth factor (EGF) (0.1 ng/ml) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

**Western blotting and antibodies.** Equal amounts of protein (50-200 µg/lane) were extracted from whole-cell lysates and were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After being transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA), the proteins were incubated with primary antibodies [FOXM1

rabbit mAb (1:500; Santa Cruz Biotechnology), E-cadherin, Vimentin, AKT, p-AKT (Ser473), p70<sup>S6K</sup> or p-p70<sup>S6K</sup> (Thr389) rabbit mAb (1:1,000; Cell Signaling Technology)], followed by appropriate HRP-conjugated secondary antibodies. Immunoreactive proteins were detected using the enhanced chemiluminescence detection kit (Thermo Scientific, Rockford, IL, USA).

**RT-PCR.** Total RNA of the cultured cells was isolated with TRIzol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA) and was then resuspended in diethylpyrocabonate (DEPC)-treated water. Reverse transcription was performed according to the manufacturer's instructions (Takara, Dalian, China). The primer sequences utilized were: FOXM1 sense, 5'-AGCGACAGGTTAAGGTTGAG-3' and antisense, 5'-GTGCTGTTGATGGCGAATTG-3'; E-cadherin sense, 5'-TGCCCAGAAAATGAAAAAGG-3' and antisense, 5'-GTGTATGTGGCAATGCGTTC-3'; Vimentin sense, 5'-GA GAACCTTTGCCGTTGAAGC-3' and antisense, 5'-CTCAAT GTCAAGGGCCATCT-3'; GAPDH sense, 5'-ACGGATTG GTCGTATTGGGCG-3' and antisense, 5'-CTCCTGGAA GATGGTGATGG-3'. The PCR products were separated by 1.5% agarose gel electrophoresis.

**Transwell migration and invasion assays.** *In vitro*, migration assays were performed in a Transwell chamber (Corning Costar, USA) containing a membrane filter (8-µm pore size). To measure cell invasion overnight, Transwell filters were coated on the lower chamber with 5 µg/ml of BioCoat Matrigel (BD Biosciences, USA) to reconstitute the matrix of the basement membrane. Cells were seeded at a density of 2.0x10<sup>4</sup>/insert in 200 µl serum-free medium and transferred to wells filled with 600 µl culture medium containing 10% FBS as a chemoattractant. After 24 h of incubation, non-invading cells on the top of the membrane were removed by scraping. Invading cells on the bottom of the membrane were fixed in 4% paraformaldehyde and stained with 0.05% crystal violet. The number of invading cells was then counted in nine random high power fields/well at x200 magnification under a light microscope.

**Transient transfection of FOXM1 siRNA.** FOXM1-specific small interfering RNA (siRNA) oligonucleotides were purchased from GenePharma (Shanghai GenePharma Co., Shanghai). The sequences of the double-stranded siRNA oligonucleotides utilized were: 5'GGACCACUUUCCCUA CUUUTT3' (sense) and 5'AAAGUAGGGAAAGUGGUC CTT3' (antisense). The negative control siRNA sequences were: 5'UUCUCCGAACGUGUCACGUTT3' (sense) and 5'ACGUGACACGUUCGGAGAATT3' (antisense). Human lung carcinoma H1975 and H1299 cells which were tested to have high FOXM1 expression were transfected with siRNA (100 nM) using the DharmaFECT4 siRNA transfection reagent (Dharmacon, Chicago, IL, USA) according to the manufacturer's protocol.

**Stable transfection of FOXM1.** Plasmid (EX-Z5438-LV135) and Lenti-Pac HIV Expression Packaging kit were purchased from GeneCopoeia Inc. (Guangzhou, China). Human lung carcinoma H1650 and A549 cells which were tested to have

Table I. Correlation between expression of FOXM1 and EMT indicator proteins and clinicopathological factors of the NSCLC patients.

Characteristics	Total (%)	High FOXM1 expression, n (%)	High E-cadherin expression, n (%)	High Vimentin expression, n (%)
Gender				
Female	30 (44.1)	18 (60.0)	15 (50.0)	10 (33.3)
Male	38 (55.9)	25 (65.8)	12 (31.6)	7 (18.4)
Age (years)				
Mean	59.44			
<60	29 (42.6)	17 (58.6)	14 (48.3)	5 (17.2)
≥60	39 (57.4)	26 (66.7)	13 (33.3)	12 (30.8)
Smoking status				
No	30 (44.1)	15 (50.0)	14 (46.7)	5 (16.7)
Yes	38 (55.9)	28 (73.7) <sup>a</sup>	13 (34.2)	12 (31.6)
Histologic characterisation				
Adenocarcinoma	33 (48.5)	20 (60.6)	14 (42.4)	6 (18.2)
Squamous carcinoma	21 (30.9)	12 (57.1)	9 (42.9)	3 (14.2)
Adenosquamous carcinoma	14 (20.6)	11 (78.6)	4 (28.6)	8 (57.1) <sup>b</sup>
Differentiation				
High/moderate	39 (57.3)	21 (53.8)	17 (43.6)	10 (25.6)
Low	29 (42.7)	22 (75.9)	10 (34.5)	7 (24.1)
TNM stage				
I+II	27 (39.7)	13 (48.1)	15 (55.6)	2 (7.4)
III+IV	41 (60.3)	30 (73.2) <sup>a</sup>	12 (29.3) <sup>a</sup>	15 (36.6) <sup>a</sup>
Lymph node metastasis				
Negative	27 (39.7)	12 (44.4)	16 (59.3)	2 (7.4)
Positive	41 (60.3)	31 (75.6) <sup>a</sup>	11 (26.8) <sup>a</sup>	15 (36.6) <sup>a</sup>

<sup>a</sup>p-value <0.05. <sup>b</sup>p-value <0.05 vs. adenocarcinoma or squamous carcinoma. FOXM1, Forkhead box M1; EMT, epithelial-mesenchymal transition; NSCLC, non-small cell lung cancer.

low FOXM1 expression were stably transfected according to the manufacturer's instructions. The presence of stable transfectants was confirmed by RT-PCR and western blotting.

**Statistical analyses.** A comparison of clinicopathologic characteristics of the patients was evaluated using the Chi-square test. Overall survival was defined as the length of time from the date of diagnosis to the date of death or the date at which patients were last known to be alive. Survival analysis data were collected until the 1st of January 2012, and the Kaplan-Meier method was used to calculate overall survival. The differences between the survival curves were analyzed using the log-rank test. Spearman rank correlation coefficients were used to quantify the correlations between the expression of FOXM1 and EMT indicator proteins. Univariate analysis was performed using the log-rank test. Multivariate analysis was performed using the Cox proportional hazards model. All factors with a value of  $p < 0.05$  by univariate analysis were included in the multivariate analysis. In the analyses,  $p < 0.05$  was considered to indicate a statistically significant result. Statistical analyses were performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA).

## Results

*Increased expression of FOXM1 is associated with EMT in NSCLC tissues.* The clinicopathological characteristics of the patients were previously reported in detail (30) and are summarized in Table I. The overall duration of the follow-up of these patients ranged from 1 to 60 months.

We sought to investigate the expression levels of the FOXM1 and EMT indicator proteins in NSCLC specimens and correlate these results with the patient clinical and pathologic findings. Immunohistochemical (IHC) analyses of tissue array slides were used to determine the expression levels of FOXM1, E-cadherin and Vimentin proteins. Either nuclear expression or mixed nuclear and cytoplasmic expression of FOXM1 in tumor cells was defined as positive immunoreactivity for FOXM1. Positive immunostaining for E-cadherin was localized to the cell membrane, whereas Vimentin was observed in the cytoplasm of cancer cells. Representative patterns of IHC staining are illustrated in Fig. 1.

As shown in Table I, high levels of expression of FOXM1, E-cadherin and Vimentin were observed in 43/68 (63.2%), 27/68 (39.7%) and 17/68 (25.0%) specimens of NSCLC,

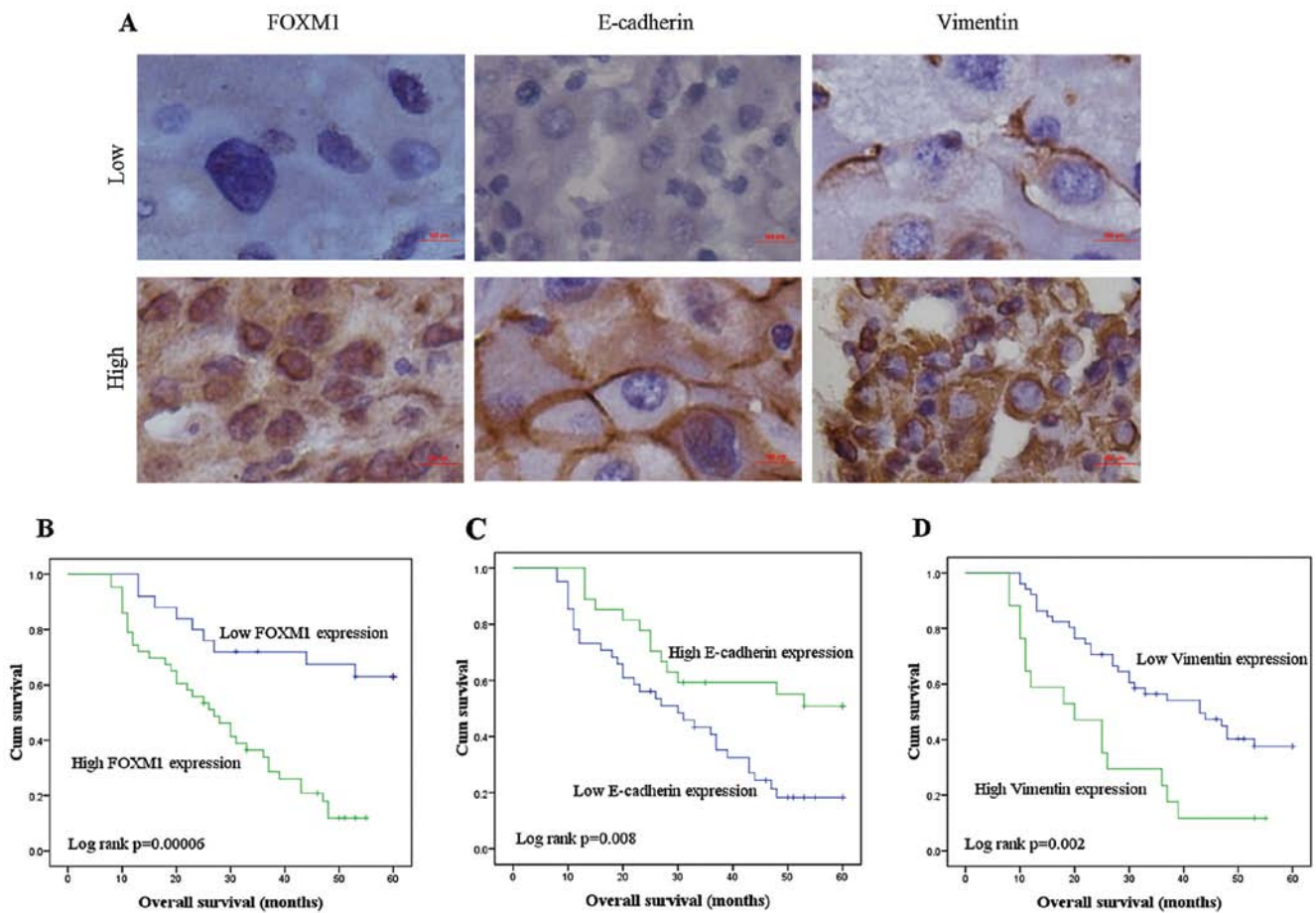


Figure 1. Representative IHC images showing expression of FOXM1 and EMT-related biomarkers in TMA sections of NSCLC and corresponding Kaplan-Meier survival curves. (A) The image shows representative NSCLC tissues exhibiting low and high levels of expression for FOXM1, E-cadherin and Vimentin. Scale bar, 100  $\mu$ m. (B-D) Kaplan-Meier survival analyses of overall survival of NSCLC patients with respect to their level of expression of FOXM1, E-cadherin and Vimentin (B, FOXM1; C, E-cadherin and D, Vimentin). FOXM1, Forkhead box M1; EMT, epithelial-mesenchymal transition; TMA, tissue microarray; NSCLC, non-small cell lung cancer.

respectively. Notably, statistically significant correlations were demonstrated between FOXM1 and the smoking history of the patients ( $p=0.044$ ), TNM stage ( $p=0.036$ ) and lymph node metastasis status ( $p=0.009$ ). Tumors from patients with a positive smoking history, advanced TNM stage and lymph node metastasis were characterized by higher FOXM1 expression compared with those without these features. Expression levels of E-cadherin and Vimentin were significantly associated with advanced TNM stage and lymph node metastasis. In addition, Vimentin expression was found to be significantly greater in lung adenosquamous carcinoma than that in squamous cell carcinoma.

*Increased expression of FOXM1 is correlated with EMT indicator proteins E-cadherin and Vimentin in NSCLC tissues.* To investigate the association between the expression of FOXM1 and EMT indicator proteins in NSCLC specimens, Spearman correlation analysis was used. As shown in Table II, a high expression level of FOXM1 was found to correlate with a loss of E-cadherin expression ( $r=-0.69$ ,  $p<0.001$ ) and anomalous immunoreactivity of Vimentin ( $r=0.370$ ,  $p=0.002$ ) in the NSCLC samples. Therefore, FOXM1 expression was significantly correlated with EMT in the NSCLC tissues.

Table II. Spearman correlation analysis between expression of FOXM1 and EMT indicator proteins in NSCLC.

	FOXM1 expression			
EMT indicator proteins	Low	High	P-value	R-value
E-cadherin expression				
Low	4	37	<0.001	-0.690
High	21	6		
Vimentin expression				
Low	24	27	0.002	0.370
High	1	16		
FOXM1, Forkhead box M1; EMT, epithelial-mesenchymal transition; NSCLC, non-small cell lung cancer.				

FOXM1, Forkhead box M1; EMT, epithelial-mesenchymal transition; NSCLC, non-small cell lung cancer.

*The expression levels of FOXM1, E-cadherin and Vimentin predict overall survival time of NSCLC patients.* Overall survival analysis was used to determine the existence of a

Table III. Univariate and multivariate analyses of overall survival.

Characteristics	Univariate analysis			Median survival (months)	Multivariate analysis		
	HR	95% CI	P-value		HR	95% CI	P-value
Gender	0.897	0.496-1.622	0.716		-	-	-
Female				36.733			
Male				35.531			
Age (years)	1.811	0.980-3.348	0.058		-	-	-
<60				42.151			
≥60				31.498			
Smoking status	1.152	0.639-2.076	0.637		-	-	-
No				37.5			
Yes				34.93			
Histologic characterisation	0.900	0.613-1.322	0.593		-	-	-
Adenocarcinoma				33.391			
Squamous carcinoma				40.899			
Adenosquamous carcinoma				35.714			
Differentiation	0.540	0.300-0.972	0.040		0.606	0.320-1.148	0.124
High or moderate				40.812			
Low				29.610			
TNM stage	2.996	1.532-5.858	0.001		1.247	0.495-3.143	0.639
I+II				45.591			
III+IV				29.733			
Lymph node metastasis	3.395	1.732-6.654	0.0001		2.595	1.034-6.510	0.042
Negative				47.113			
Positive				28.548			
FOXM1 expression	4.099	1.934-8.688	0.00023		2.950	1.036-8.397	0.043
Low				47.645			
High				28.610			
E-cadherin expression	0.429	0.223-0.824	0.001		1.181	0.500-2.789	0.704
Low				31.297			
High				43.344			
Vimentin expression	2.596	1.386-4.864	0.003		1.287	0.642-2.580	0.477
Low				40.012			
High				23.882			

HR, hazard ratio; CI, confidence interval; FOXM1, Forkhead box M1.

correlation between the expression of FOXM1 and EMT indicator proteins and patient prognosis. Kaplan-Meier survival curves for overall survival with respect to FOXM1 and EMT indicator protein expression are shown in Fig. 1. These plots demonstrated that NSCLC patients expressing low levels of FOXM1 and Vimentin but high levels of E-cadherin survived significantly longer than patients with NSCLC expressing high FOXM1 and Vimentin but low E-cadherin levels.

In addition, a Cox proportional hazards model was applied to estimate the effect of FOXM1 and EMT indicator proteins on patient survival. Univariate Cox regression analysis identified tumor differentiation, TNM stage, lymph node metastasis, and expression of FOXM1, E-cadherin and Vimentin as significant prognostic factors (Table III). The crude hazard ratio

(HR) of NSCLC with high FOXM1 expression compared with low FOXM1 expression was 4.099 (95% confidence interval 1.934-8.688,  $p=0.00006$ ). In other words, high FOXM1 expression in NSCLC patients increased the likelihood of lung cancer-related death 4-fold above that of a low FOXM1 status. Using multivariable analysis, lymph node metastasis and FOXM1 were found to be statistically significant independent prognostic factors for overall survival ( $p=0.042$  and  $p=0.043$ , respectively). These findings suggest that FOXM1 expression behaves as an independent and statistically significant predictor for poor patient survival.

*Stable overexpression of FOXM1 protein induces EMT in NSCLC cells.* Our data provide clinical evidence supporting

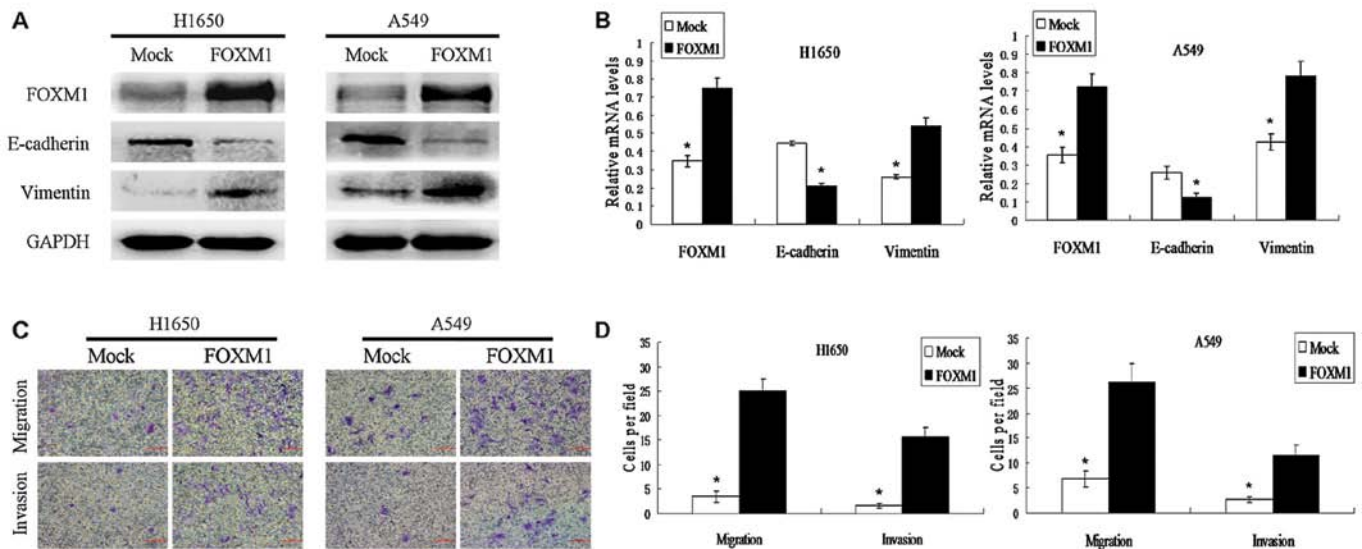


Figure 2. Effects of lentiviral-mediated FOXM1 overexpression on EMT and the invasive capacity of NSCLC cells. (A) H1650 and A549 cells transfected with a control or FOXM1-specific lentivirus were harvested, lysed and then subjected to SDS-PAGE, transferred to membranes, and blotted with the indicated antibody with GAPDH as a loading control. (B) Quantification of the target mRNA bands relative to GAPDH is shown in the histograms. (C) Effects of FOXM1 overexpression on the invasive capacity of H1650 and A549 cells by Transwell assay. The invasive cells were stained and counted under a microscope. Scale bar, 500  $\mu$ m. (D) Quantitative results for the transmembrane ability of each group of cells. \* $p$ <0.05. FOXM1, Forkhead box M1; EMT, epithelial-mesenchymal transition; NSCLC, non-small cell lung cancer.

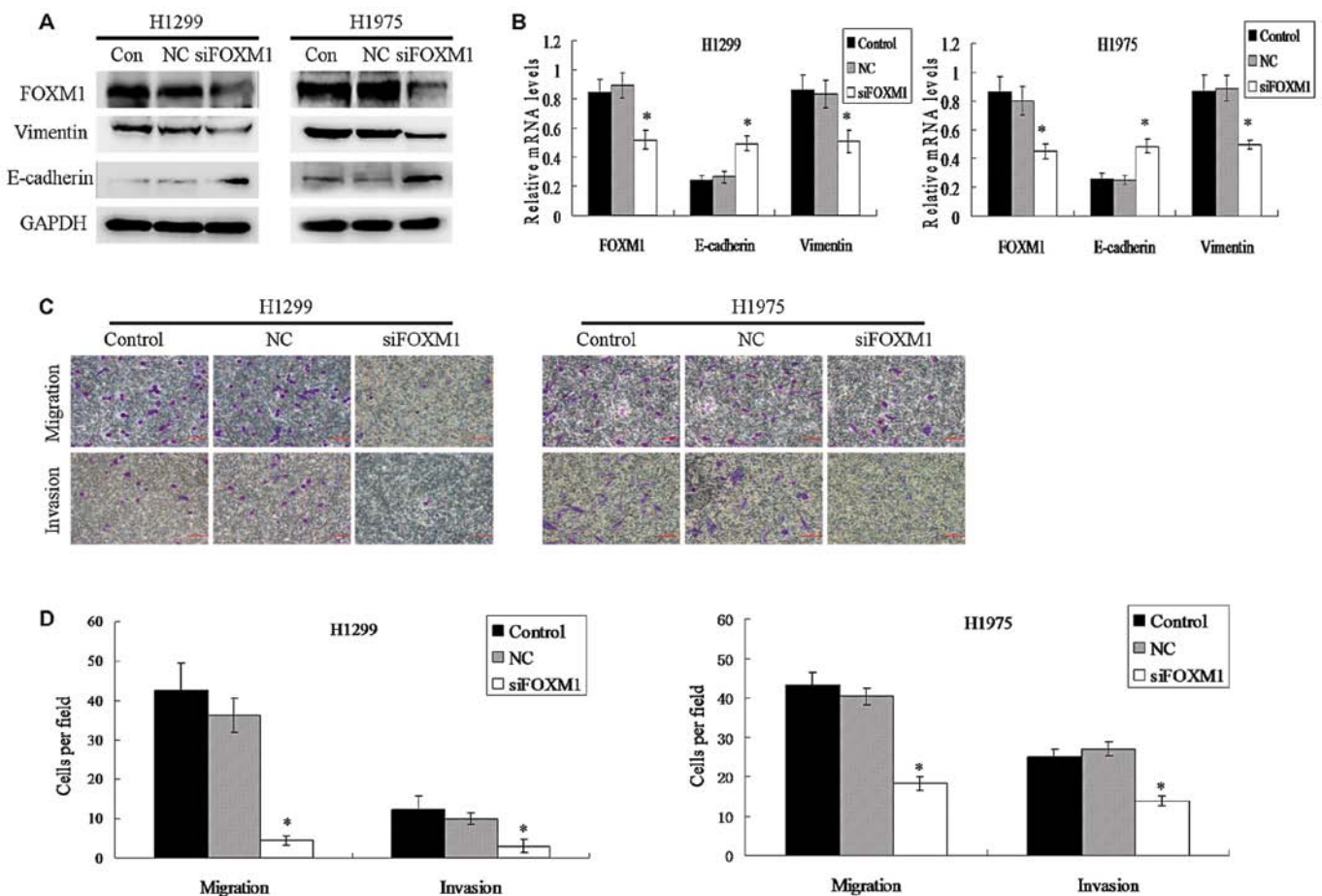


Figure 3. Effects of siRNA-mediated FOXM1 inhibition on EMT and the invasive capacity of NSCLC cells. (A) One pair of siRNA specifically against FOXM1 or negative control siRNA (NC) was transiently transfected into H1299 or H1975 cells. Two days after transfection, FOXM1 and EMT indicator proteins were detected by western blotting with GAPDH used as a loading control. (B) Quantification of the target mRNA bands relative to GAPDH is shown in the histograms. (C) Effects of siRNA-mediated FOXM1 inhibition on the invasive capacity of H1299 and H1975 cells by Transwell assay. The invasive cells were stained and counted under a microscope. Scale bar, 500  $\mu$ m. (D) Quantitative results for the transmembrane ability of each group of cells, \* $p$ <0.05. FOXM1, Forkhead box M1; EMT, epithelial-mesenchymal transition; NSCLC, non-small cell lung cancer; siRNA, small interfering RNA.



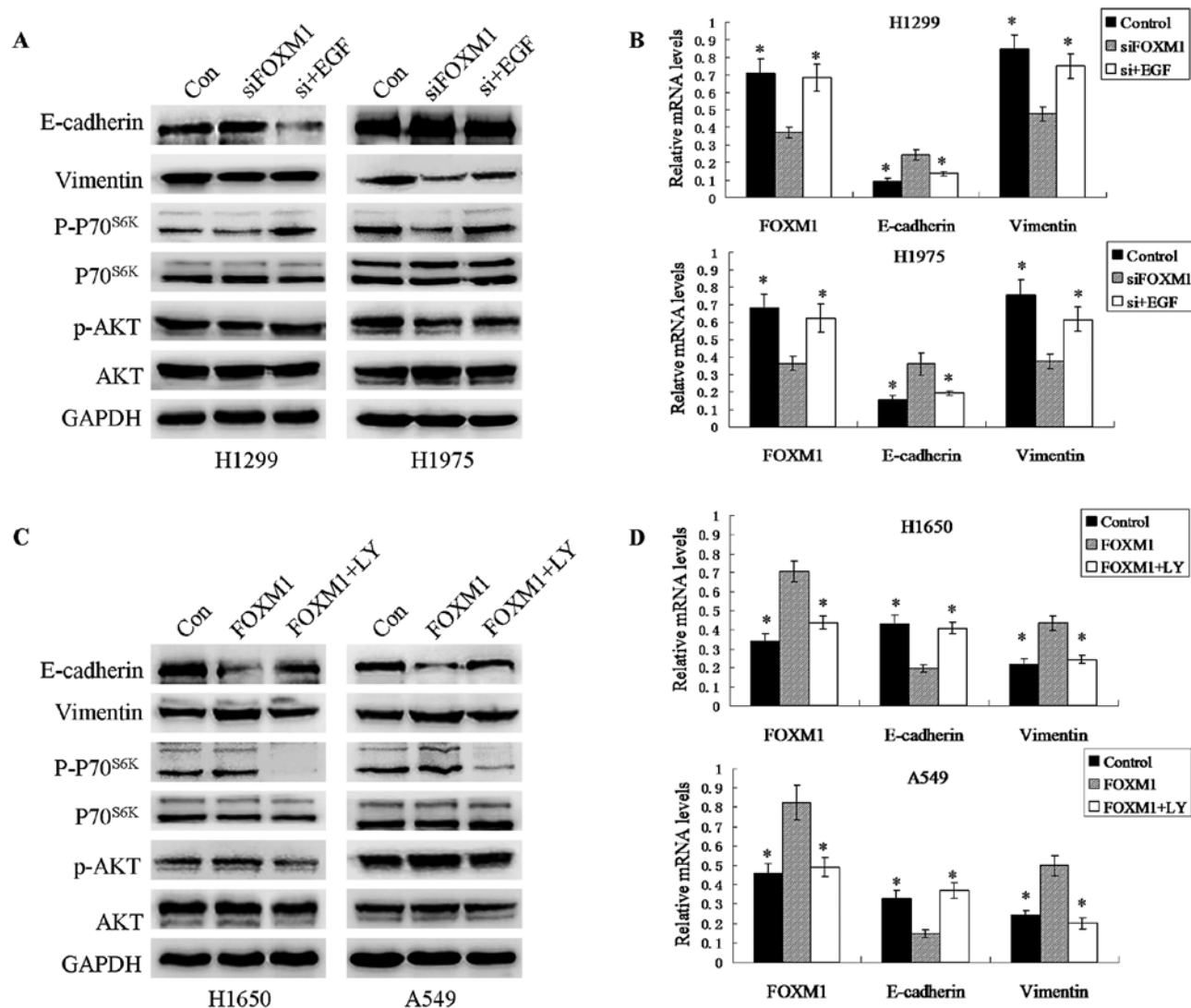


Figure 4. Effects of inhibition and activation of the AKT/p70<sup>S6K</sup> pathway on EMT of NSCLC cells. H1299 and H1975 cells transiently transfected with siRNA specifically against FOXM1 were treated with EGF (0.1 ng/ml) for 48 h. Effect of the kinase activator on the expression of EMT indicator proteins was detected by (A) western blotting and (B) RT-PCR, \*p<0.05 vs. siFOXM1 or FOXM1. H1650 and A549 cells transfected with the FOXM1-specific lentivirus were treated with LY294002 (LY) (10  $\mu$ mol/l) for 48 h. Effect of the kinase inhibitor on the expression of EMT indicator proteins was detected by (C) western blotting and (D) RT-PCR. EMT, epithelial-mesenchymal transition; NSCLC, non-small cell lung cancer; siRNA, small interfering RNA; EGF, epidermal growth factor.

the hypothesis that high FOXM1 expression is associated with EMT and poor survival of patients with NSCLC. Furthermore, FOXM1 has been implicated in the regulation of the EMT process in pancreatic cancer cells (31). To test whether FOXM1 expression induces EMT in NSCLC cells, human lung carcinoma H1650 and A549 cells were stably transfected with a lentiviral vector expressing FOXM1. Following transfection, protein and mRNA levels of E-cadherin were found to be decreased while those of Vimentin were increased in these cells (Fig. 2A and B). To further characterize the EMT process in FOXM1-expressing cells, the migration capacity of FOXM1-transfected cells and control lentivirus-transfected (mock) cells were compared using Transwell chambers. The elevated expression of FOXM1 promoted the migration and invasion of transfected cells when compared with the mock cells (Fig. 2C and D). These data suggest that FOXM1 may promote metastasis by inducing the EMT process in NSCLC cell lines.

**Knockdown of FOXM1 expression inhibits the EMT process of NSCLC cells.** To test whether FOXM1 expression was necessary for the EMT process of NSCLC cells, the human NSCLC cell lines H1299 and H1975 were transiently transfected with negative control siRNA or FOXM1-specific siRNA to suppress the expression of FOXM1. Knockdown of FOXM1 gene expression increased the expression of E-cadherin while decreasing the expression of Vimentin both at the protein and mRNA levels in the transfected cells (Fig. 3A and B). As shown in Fig. 3C and D, knockdown of FOXM1 markedly reduced the migration and invasion of the transfected cells. These results suggest that FOXM1 expression is critical for the EMT process in lung cancer cells.

**Overexpression and knockdown of FOXM1 expression regulate EMT through activation and inhibition of the AKT/p70<sup>S6K</sup> signaling pathway.** To explore the mechanism whereby FOXM1 expression enhances EMT, we examined the effects

of FOXM1 on the expression and phosphorylation of AKT and p70<sup>S6K</sup>. We found that knockdown of endogenous FOXM1 inhibited the phosphorylation levels of AKT and p70<sup>S6K</sup> but not total AKT/ p70<sup>S6K</sup>. Conversely, overexpression of FOXM1 induced the opposite results.

To further investigate whether the activation of AKT pathway is responsible for the FOXM1-induced EMT in NSCLC, an inhibitor (LY294002) and an activator (EGF) of the PI3K/AKT pathway were used in experiments based on the observation that overexpression and knockdown of FOXM1 can activate and inhibit this pathway, respectively. As shown in Fig. 4, FOXM1-expressing cells treated with the inhibitor LY294002 showed reduced expression of Vimentin but increased E-cadherin expression. In contrast, EMT was promoted in siRNA-transfected cells incubated with EGF for 48 h. These results suggest that activation of the AKT/p70<sup>S6K</sup> pathway plays an important role in FOXM1-induced EMT in NSCLC.

## Discussion

In the present study, we demonstrated that high levels of expression of FOXM1 and EMT indicator proteins are significantly associated with several clinicopathologic variables. We also demonstrated, for the first time, the existence of a significant association between FOXM1 and EMT indicator protein expression. Additionally, the present study demonstrated that FOXM1 expression may be an independent prognostic indicator for overall survival of patients with NSCLC. Furthermore, our results suggest that FOXM1 expression may promote NSCLC metastasis by inducing EMT of tumors cells via the activation of the AKT/p70<sup>S6K</sup> pathway in lung cancer cells.

As previously mentioned, the existence of a significant association between FOXM1 expression and poor prognosis has been reported in many human types of cancers, including lung cancer (6,12,32). In agreement with these reports, we found that FOXM1 is an independent prognostic indicator for overall survival of NSCLC patients. Our results also suggest that FOXM1 expression in tumor specimens is positively associated with TNM stage and the presence of lymph node metastasis. However, Liu *et al* (32) reported no association between FOXM1 expression and the clinical parameters studied, which raises questions about how tumor specimens were obtained and how IHC expression was analyzed.

There is considerable evidence that the presence of EMT signifies reduced survival of lung cancer patients (33-36). Previous studies reported that negative or low E-cadherin expression ranged from 46 to 75.21% in NSCLC specimens (37-40), which is similar to the low E-cadherin expression level of 60.3% found in the present study. Our results also showed that Vimentin expression was significantly associated with the presence of lymph node metastasis, TNM stage and the histologic type of the tumor. In contrast with our results, Dauphin *et al* (41) did not identify any association between Vimentin expression and lymph node metastasis. These differences may reflect the different study populations and varied clinical data quality.

To the best of our knowledge, this is the first report that high FOXM1 expression is significantly associated with EMT in NSCLC specimens. To extend the above results, we studied

overexpression or knockdown of FOXM1 in lung cancer cell lines and found that FOXM1-mediated EMT results in the invasion and migration *in vitro*. Notably, Xu *et al* (12) reported that levels of E-cadherin and ZO-1 were reduced and that Vimentin and N-cadherin expression levels were elevated in NSCLC cells expressing high levels of FOXM1. These results suggest that FOXM1 expression is associated with poor prognosis by regulating EMT in NSCLC.

Until recently, the precise molecular regulation of FOXM1 on EMT and metastasis was unclear. Previous research has shown that AKT has a distinct role in regulating EMT and cell migration (42). Furthermore, FOXM1 has been shown to activate the AKT-Snail pathway to promote cell migration and invasion in hepatocellular carcinoma (29) and early stage cervical cancer (11), while its role in NSCLC has not been reported. The present study demonstrated that FOXM1 activates the AKT/p70<sup>S6K</sup> signaling pathway thereby promoting EMT transition by enhancing cell migration and the invasive capacity in NSCLC. Furthermore, our research team is currently exploring the possible mechanisms involved in the regulation of PI3K/AKT by FOXM1.

In summary, our results suggest that FOXM1 expression is associated with a poor prognosis and EMT in NSCLC. In cell culture experiments, FOXM1 overexpression promoted migration and invasion by inducing the EMT of lung cancer cells via activation of the AKT/p70<sup>S6K</sup> pathway. The present study raises the possibility that FOXM1 may be a potential target for cancer therapy as it plays a crucial role in tumor migration and invasion.

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