Transcription Repressor *Slug* Promotes Carcinoma Invasion and Predicts Outcome of Patients with Lung Adenocarcinoma

Jin-Yuan Shih,[1] Meng-Feng Tsai,[1] Tzu-Hua Chang,[1] Yih-Leong Chang,[2] Ang Yuan,[3] Chong-Jen Yu,[1] Shin-Bey Lin,[1] Geou-Yarh Liou,[1] Meng-Larn Lee,[5] Jeremy J.W. Chen,[6,8] Tse-Ming Hong,[5] Shuenn-Chen Yang,[5] Jen-Liang Su,[7] Yung-Chie Lee,[4] and Pan-Chyr Yang[1,5,6,9]

Abstract

Purpose: In a previous genome-wide gene expression profiling analysis using an invasion cancer cell lines model, we have identified *Slug* as selectively overexpressed in the highly invasive cancer cells. Here, we investigated the clinical significance of *Slug* in lung adenocarcinoma and the role of *Slug* in the process of cancer cell invasion and metastasis.

Experimental Design: Real-time quantitative reverse transcription-PCR was used to investigate *Slug* mRNA in surgically resected lung adenocarcinoma of 54 patients and its correlation with survival. We overexpressed *Slug* in a lung adenocarcinoma cell line with very low *Slug* levels and investigated the *in vitro* and *in vivo* effects of *Slug* expression.

Results: High expression of *Slug* mRNA in lung cancer tissue was significantly associated with postoperative relapse ($P = 0.03$) and shorter patient survival ($P < 0.001$). The overexpression of *Slug* enhanced xenograft tumor growth and increased microvessel counts in angiogenesis assay. Both inducible and constitutive overexpression of *Slug* suppressed the expression of E-cadherin and increased the *in vitro* invasive ability. Zymography revealed increased matrix metalloproteinase-2 activity in *Slug* overexpressed cells. ELISA, reverse transcription-PCR, and immunohistochemistry confirmed the increase of matrix metalloproteinase-2 proteins and mRNA in *Slug* overexpressed cells and xenograft tumors.

Conclusions: *Slug* expression can predict the clinical outcome of lung adenocarcinoma patients. *Slug* is a novel invasion-promoting gene in lung adenocarcinoma.

Lung cancer is the leading cause of cancer death worldwide. Metastasis is the most common cause of death in lung cancer patients and is a major obstacle to the successful treatment. The spread of tumor cells from a primary tumor to the secondary sites within the body is a complicated process involving cell proliferation and migration, degradation of basement membrane, invasion, adhesion, and angiogenesis (1). A variety of positive and negative factors are involved in this highly sophisticated process of metastasis (2). Current clinical means cannot accurately identify those patients who will develop metastasis. To develop effective new strategies for the prediction, diagnosis, and treatment of metastasis of lung cancer, molecular mechanisms controlling metastasis must be identified (3).

Cancers are a mass of heterogeneous neoplastic cells with different properties, including metastatic potential (4). During cancer development, some tumor cells acquire metastatic phenotypes, overexpression of metastasis-promoting genes or loss of expression of metastasis-suppressing genes. Recently, several groups have successfully used gene expression profiling techniques and model systems with different invasive or metastatic ability to identify genes that correlate with invasiveness or metastatic potential (5–9).

Multiple rounds of *in vitro* and *in vivo* selection of subclones of cancer cells originating from the same primary lung adenocarcinoma may result in the establishment of several phenotypically stable cell lines that differ strongly in their invasive and metastatic potential both *in vitro* and *in vivo* (10, 11). We have established previously a panel of lung cancer cell line with different invasion capability (CL1-0, CL1-1, CL1-5, and CL1-5-F4 in order of increasing invasion activity; refs. 7, 10). CL1-5 and CL1-5-F4 cells have a high potential of invading through natural or artificial basement membranes and form distant metastases when injected into severe combined immunodeficient (SCID) mice. Conversely, CL1-0 cells have a low capability of invading through basement membranes and do not form metastases in SCID mice. By using cDNA

Authors' Affiliations: Departments of [1]Internal Medicine, [2]Pathology, [3]Emergency Medicine, and [4]Surgery, National Taiwan University Hospital; [5]Institute of Biomedical Sciences, Academia Sinica; [6]NTU Center for Genomic Medicine and [7]Institute of Toxicology, National Taiwan University College of Medicine; [8]Institutes of Biomedical Sciences and Molecular Biology, National Chung-Hsing University; and [9]National Health Research Institute, Taipei, Taiwan

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Note: J-Y. Shih and M-F. Tsai contributed equally to this work.

Requests for reprints: Pan-Chyr Yang, Department of Internal Medicine, National Taiwan University Hospital, No. 7 Chung-Shan South Road, Taipei 100, Taiwan. Phone: 886-2-23562905; Fax: 886-2-23582867; E-mail: pcyang@ha.mc.ntu.edu.tw.

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microarray, we were able to identify invasion- and metastasis-associated genes on a genome-wide scale in model lung cancer cell lines (7, 8). Among these metastasis-associated genes, we identified one candidate gene, *Slug*, whose mRNA expression showed a positive correlation with invasive potential in this panel of cell lines.

Slug is a member of the *Snail* family of zinc finger transcription factors. They recognize the CAGGTG-binding site identified first in *Drosophila* and then in mammals and humans (12, 13). The *Snail* family has been shown to participate in mesoderm formation, neural crest cell formation and migration, cell differentiation, cell adhesion, cell invasion, cell cycle regulation, and antiapoptosis (14–18). Although the *Snail* family has been supposed to play a role in epithelial-mesenchymal transitions (EMT) concomitantly with down-regulation of E-cadherin expression, most of the studies are about the functions of *Snail* (19), and the effect of *Slug* expression in cancer cells has not been clarified. The aim of this study was to evaluate the clinical significance of *Slug* mRNA expression in lung adenocarcinoma patients and the role of Slug in cancer cell invasion and metastasis.

Patients and Methods

Patients and specimens. Fifty-four patients who underwent resection for adenocarcinoma lung cancer at the National Taiwan University Hospital between September 1994 and December 1996 were studied. This study was approved by the institutional review board of National Taiwan University Hospital. Written informed consent was obtained from all patients. Specimens of lung cancer tissue obtained at surgery were immediately snap frozen in liquid nitrogen and stored until use.

The histologic classification of these tumors was based on the WHO criteria (20). Pathologic staging was done according to the tumor-node-metastasis system for lung cancer staging (21). The patients consisted of 24 men and 30 women (mean age, 60.2 ± 10.8 years). The surgicopathologic stage of disease was I in 19 patients, II in 7 patients, III in 20 patients, and stage IV in 8 patients. Tumor status was T_1 in 12 patients, T_2 in 26 patients, T_3 in 13 patients, and T_4 in 3 patients. Twenty-four patients had no lymph node metastasis (N_0), whereas 30 had regional or mediastinal lymph node metastasis (N_1 in 11 patients and N_2 in 19 patients). The three T_4 cases included two multifocal adenocarcinomas within one pulmonary lobe and one with vertebral body invasion. Stage IV cases were multifocal adenocarcinomas involving two pulmonary lobes. Among these 54 patients, no patients received neoadjuvant chemotherapy or radiotherapy before surgery. Twenty patients had adjuvant chemotherapy and/or radiotherapy. After cancer recurrence, the treatment was determined by individual physicians caring for their patients.

Real-time quantitative reverse transcription-PCR. Total mRNA was extracted from resected cancer tissue using a RNA extraction kit (RNeasy Mini kit; Qiagen, Valencia, CA). The quality of the RNA samples was determined by electrophoresis through agarose gels and staining with ethidium bromide. The primers used for quantitative reverse transcription-PCR (RT-PCR) of *Slug* mRNA were forward primer 5′-AGAACT-CACACGGGGGAGAAG-3′ and reverse primer 5′-CTCAGATTTGAC-CTGTCTGCAAA-3′. The sequence of the probe used to detect and quantify the RT-PCR product was carboxyfluorescein 5′-TTTTTCTTG-CCCTCACTGCAACAGAGC-3′ *N,N,N′,N′*-tetramethyl-6-carboxyrhodamine. The primers and probe used for quantitative RT-PCR of *TATA box–binding protein (TBP)* mRNA (internal control) were according to the publication of Bieche et al. (22). The identities of PCR products were confirmed by DNA sequencing. The reaction conditions were as described previously (8). The fluorescence emitted by the reporter dye was detected online in real-time using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA).

Cells lines, cloning of* Slug, *and transfection. Human lung adenocarcinoma cell lines CL1-0, CL1-1, CL1-5, and CL1-5-F4 were grown in RPMI 1640 (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS) and 2 mmol/L L-glutamine at 37°C, 20% O_2, and 5% CO_2 (7, 10). A cDNA encoding the entire human *Slug* coding region was amplified from the cDNA of CL1-5 by PCR. The *Slug* cDNA was cloned into a TA vector according to the manufacturer's instructions (pGEM-T-Easy Cloning kit; Promega, Madison, WI). Sequence analysis showed 100% homology to the published sequence (23). pCIneo-*Slug* was created by inserting the *Slug* cDNA fragment into a pCI-neo expression vector (Promega) and used for transfection and expression of Slug in CL1-0 cells.

pCIneo-*Slug* plasmid was transfected into 70% confluent CL1-0 cells with LipofectAMINE method (Invitrogen). Gentamicin (G418; Invitrogen) was added to 500 μg/mL for the selection of stable transfectants. Clones of resistant cells were isolated. Slug4 and Slug6 were selected as representatives of high-Slug overexpression clone and moderate-Slug expression clone, respectively. The mock vector-transfected cells were used in bulk for the control. RNA isolated from stable transfectant clones was treated with DNase I before real-time quantitative RT-PCR was done.

We further constructed pTRE2-*Slug* vector for inducible expression of *Slug* in CL1-0. CL1-0 was transfected pTet-Off vector (Clontech, Palo Alto, CA) with LipofectAMINE method. G418-resistant cells were isolated. The picked clones were screening by transient transfection with pTRE2-Luc for clones with low background and high induction of luciferase in response to doxycycline (Sigma, St. Louis, MO) 1 μg/mL. CL1-0 Tet-Off cell line was selected and transfected with pTRE2-*Slug* or pTRE2 vector concomitant with pTK-Hyg. Hygromycin-resistant cells were isolated. The expression of *Slug* in resistant clones was analyzed by real-time quantitative RT-PCR in each clone. Clone Slug33 was selected because it had very low *Slug* expression when cultured in doxycycline and had high expression of *Slug* when doxycycline was removed. The pTRE2 and pTK-Hyg vector-transfected pool was named as T2, which acted as a control clone.

Northern hybridization analysis and reverse transcription-PCR. RNA from cell lines was extracted using Trizol reagent (Invitrogen). Northern hybridization was done as described previously (8). Briefly, each lane on formaldehyde gels was loaded with 20 μg total RNA. After electrophoresis, the gels were blotted onto nylon membranes. After cross-linking and prehybridization, they were hybridized with ^{32}P-labeled DNA probes. The membranes were washed and then exposed to X-ray film overnight at −70°C. The amount of RNA in each lane was measured by comparing with the signal intensity of Gβ-like probe (a housekeeping gene used as an internal control for RNA quantity; ref. 24).

To analyze the expression of *Slug, Snail, E-cadherin*, and *matrix metalloproteinase-2* (*MMP-2*) in cell lines, RT-PCR was done. Polyadenylated RNA was isolated from the different tumor cell lines or stable transfectant clones and treated with DNase I before cDNA synthesis. The mRNA was reverse transcribed using SuperScript II reverse transcriptase (Invitrogen). PCR for *Slug, Snail, E-cadherin*, and *MMP-2* were done at an annealing temperature of 60°C. Gβ-like was amplified after 28 cycles at an annealing temperature of 56°C. Primer sequences were as follows: *Slug* forward 5′-GAGCATACAGCCCCAT-CACT-3′ and reverse 5′-GCAGTGAGGGCAAGAAAAAG-3′, *Snail* forward 5′-GCGAGCTGCAGGACTCTAAT-3′ and reverse 5′-TCCAAG-GAAGAGGCTGAAGTA-3′, *E-cadherin* forward 5′-TTTGTACAGAT-GGGGTCTTGC-3′ and reverse 5′-CAAGCCCACTTTTCATAGTTCC-3′, and *MMP-2* forward 5′-ATGGCGATGGATACCCCTTT-3′ and reverse 5′-ACGGACCACTTGGCCTTCT-3′.

In vitro *cell invasion and migration assay.* *In vitro* Matrigel invasion assays were done using 6.5-mm Costar transwell chambers (8-μm pore size; Corning, NY). The Transwell filters were coated with appropriate Matrigel (Becton Dickinson, Franklin Lakes, NJ). After the Matrigel solidified at 37°C, 1×10^5 cells were seeded onto the Matrigel. After 24-hour incubation, the filter was gently removed from the chamber and the noninvasive cells on the upper surface were removed by wiping

with a cotton swab. The cells that invaded the Matrigel and attached to the lower surface of the filter were fixed with methanol and stained with Giemsa solution. The number of cells attached to the lower surface of the polycarbonate filter was counted at ×400 magnification under a light microscope. Each type of cell was assayed in triplicate.

Cell motility was assessed using a scratch wound assay. The cells were seeded into six-well tissue culture dishes at a concentration of 1×10^6 and cultured in medium containing 10% FBS to nearly confluent cell monolayers, which were then carefully wounded using a cell scraper. Any cellular debris was removed by washing with PBS. After making wounds, the cultures were incubated at 37°C and photographed immediately ($t = 0$) and 24 hours later. Migration was evaluated by the number of cell migration into the cell-free zone. The experiments were repeated in quadruplicate wells thrice.

Cell proliferation assay. The cells were seeded onto 96-well plates at 4,000 per well in culture medium (100 μL). After culturing for various durations, cell numbers were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described previously (8). Briefly, at each time point, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (10 μL; 5 mg/mL) was added to each well and incubated for 4 hours at 37°C. The reaction was stopped by adding 100 μL of 0.04 N HCl in isopropanol to each well, with vigorous mixing to solubilize colored crystals produced by the reaction. The absorbance at 570 nm to absorbance at 630 nm as reference wave was measured by a multiwell scanning spectrophotometer. Each data point is the average of six determinations and each experiment was repeated thrice.

Cloning of E-cadherin and transient transfection. The full-length human *E-cadherin* cDNA was cloned into the constitutive mammalian expression vector pCDNA3 (Invitrogen). The resultant plasmid was named pCDNA3-*ECad*. Subsequently, the CL1-5 cells were transfected with pCDNA3-*ECad* and pCDNA3 empty vector using LipofectAMINE method. Twenty-four hours after transfection, the cells were collected for *in vitro* invasion assay. For Western blotting, these cells were lysed 48 hours after transfection and subjected to SDS-PAGE. The antibodies used for Western blot analyses were anti-E-cadherin monoclonal antibody (sc-8426, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti-α-tubulin monoclonal antibody (CP06, Calbiochem, San Diego, CA) and an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ) as substrate.

Zymography for gelatinase. Zymographic analysis of gelatinase activity in secreted medium was done in 10% polyacrylamide gels containing 0.1% gelatin (25). Cells were cultured in RPMI 1640 containing 10% FBS. After 2 hours, cells were washed extensively and changed to serum-free RPMI 1640. After an overnight incubation, media were collected and mixed with sample buffer for electrophoresis. After electrophoresis, the SDS was removed from the gel by incubating in 2.5% (v/v) Triton X-100 for 30 minutes. The gels were then incubated at 37°C overnight in development buffer [50 mmol/L Tris-HCl (pH 7.6), containing 0.2 mol/L NaCl, 5 mmol/L $CaCl_2$] and stained with 40% methanol/10% glacial acetic acid containing 0.5% (w/v) Coomassie brilliant blue G-250 for 20 minutes.

ELISA assay for matrix metalloproteinase-2. Commercial ELISA kit (Calbiochem) was used to analyze the cellular level of MMP-2. Briefly, anti-MMP-2 monoclonal antibody was precoated on the 96-well microtiter plate. The second anti-MMP-2 antibody was added to each well. The standard or unknown sample (100 μL) was then added to the well. The plate was then incubated at room temperature for 2 hours and the wash was repeated. A substrate solution was then added to all wells and incubated for 30 minutes. At this point, a stop solution was added to all wells. Color development and intensity of the color were measured using an ELISA plate reader. A standard curve was prepared, plotting the absorbance versus the concentration of the cytokine expressed as picogram per milliliter in the original samples.

In vivo *murine angiogenesis assays.* All animal work was done under protocols approved by the Institutional Animal Care and Use Committee of the College of Medicine, National Taiwan University. The effect of Slug on the *in vivo* angiogenesis was evaluated by the murine angiogenesis model using Matrigel plug assay as described by Passaniti et al. with modification (26). Briefly, the 1×10^6 cells were mixed with Matrigel (8.0 mg/mL; 0.4 ml), and the Matrigel plug was injected s.c. into each SCID mouse (supplied by the animal center in the College of Medicine, National Taiwan University). In total, three mice were injected with Matrigel plug containing mock cells and three mice were injected with Matrigel plug containing Slug4 cells. After 10 days, mice were sacrificed, and the Matrigel plug was removed to assess the angiogenesis activity. For histologic analysis, the Matrigel plug, in combination with surrounding skin and soft tissue, was embedded in ornithine carbamyl transferase and frozen in −80°C. Sections (5 μm) were then stained with rat anti-mouse monoclonal antibody CD31 (BD PharMingen, Bedford, MA) for mouse endothelial cell staining. The microvessels surrounding the tumor nest were calculated at ×200 field, and three fields of area with most intense neovascularization surrounding the tumor nest were counted.

Tumorigenicity in severe combined immunodeficient mice. Six-week-old SCID mice were housed in an isolator and *ad libitum* fed with autoclaved food. For tumor growth in animals, cancer cells were trypsinized, washed, centrifuged, and resuspended in HBSS (Invitrogen). A total volume of 0.2 mL containing 5×10^6 cells was s.c. injected on the dorsal region of each animal. Control cells (mock) were injected on the left side and *Slug* transfectant (Slug4) was injected on the right side. A total of six mice were studied. Injected mice were examined every 7 days for tumor appearance and tumor volumes were estimated from the length (a) and width (b) of the tumors using the formula: $V = ab^2 / 2$ (27). After 49 days, animals were sacrificed, and tumors were confirmed by histologic examination. For histologic examination, tissues were fixed in PBS/10% formalin and embedded in paraffin. From each paraffin block, three consecutive sections were cut, which were stained with H&E, anti-MMP-2 antibody (Daiichi Fine Chemical Co. Ltd., Tokyo, Japan), and anti-E-cadherin antibody (Takara Shuzo Co. Ltd., Kyoto, Japan). Immunohistochemical staining was carried out using a modified avidin-biotin peroxidase complex method. Intensity of immunohistochemical staining was scored from 0 to 2+: 0, no staining of cancer cells; 1+, heterogenous staining of cancer cells; and 2+, homogeneous staining of cancer cells.

Statistical analysis. Where appropriate, the data are presented as the mean ± SD. All statistical analyses were done with SPSS version 11.0 (SPSS, Inc., Chicago, IL). Continuous data were compared using Student's t test. The Fisher's exact test was used to compare the clinicopathologic characteristics of patients with high or low *Slug* mRNA expression. Survival curves were plotted by the Kaplan-Meier method and compared by log-rank test. For multivariate analysis of survival, Cox regression model procedure was done. Ps < 0.05 were considered statistically significant.

Results

Slug *mRNA expression in lung cancer tissue correlates with postoperative relapse and survival of lung cancer patients.* Real-time quantitative RT-PCR was used for quantifying transcript copy number of *Slug*. The threshold cycle (CT) was defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe exceeds a fixed threshold above baseline. For a chosen threshold, a smaller starting copy number results in a higher CT value. In this study, we used *TBP* mRNA as an internal control. The relative amounts of tissue *Slug* mRNA, standardized against the amount of *TBP* mRNA, were expressed as $-\Delta CT = -[CT_{Slug} - CT_{TBP}]$. The ratio of *Slug* mRNA copies to *TBP* mRNA copies was then calculated as $2^{-\Delta CT} \times K$, where K is a constant (8). Of the 54 tumor samples, the $-\Delta CT$ value ranged from −4.69 to 4.96 with a median of −0.09. The median value was used to classify patients as *Slug* high-expression group or *Slug* low-expression group. There were no differences in age,

gender, disease stage, tumor status, and lymph node metastasis between the two groups (Table 1). Counter to expectations, *Slug* expression was not associated with presence of lymph node metastasis or stage. The median duration to postoperative recurrence was shorter in the *Slug* high-expression group (11.0 months; 95% confidence interval, 8.7-13.4 months) than in the *Slug* low-expression group (27.0 months; 95% confidence interval, 0-54.8 months; $P = 0.03$, log-rank test; Fig. 1A). The *Slug* high-expression group (median survival, 14.9 months; 95% confidence interval, 4.9-24.9 months) had a significantly shorter survival than the *Slug* low-expression group (median survival, 41.8 months; $P < 0.001$, log-rank test; Fig. 1B).

Univariate analysis showed that *Slug* mRNA expression, disease stage, tumor status, nodal status, adjuvant chemotherapy and/or radiotherapy, and age were prognostic factors for relapse and survival. Multivariate analysis using the Cox regression model, *Slug* mRNA expression ($P = 0.02$), stage of disease ($P = 0.01$), and age ($P = 0.002$) were the significant prognostic factors for survival, whereas *Slug* mRNA expression ($P = 0.002$) and stage of disease ($P < 0.001$) were significant factors for predicting recurrence.

Identification of differentially expressed* Slug *mRNA by cDNA microarray. Previously, we used cDNA microarray with colorimetric detection to identify differentially expressed genes among lung cancer cell lines (CL1-0, CL1-1, CL1-5, and CL1-5-F4) with varying degrees of invasive properties (7, 8). We found that the mRNA expression of *Slug* correlated positively with cell line invasiveness (Fig. 2A). Northern hybridization confirmed that the level of *Slug* expression was drastically increased in CL1-5 and CL1-5-F4 relative to CL1-0 and CL1-1 (Fig. 2B). Real-time quantitative RT-PCR of *Slug* in cell lines also confirmed the result of cDNA microarray (Fig. 2C). Then, we checked the Snail expression in this panel of cell lines by RT-PCR and found the amount of *Snail* mRNA was equal in the series of cell lines (data not shown). Therefore, we focused our study on the function of *Slug* in cancer invasion.

Table 1. Clinicopathologic characteristics of tumors with high and low *Slug* mRNA expression

Characteristics	*Slug* mRNA		*P*
	$-\Delta CT < -0.09$, no. patients	$-\Delta CT > -0.09$, no. patients	
Mean age (y)	60 ± 11	60 ± 11	0.897*
Sex			
Male	11	13	0.786
Female	16	14	
Stage			
I-II	13	13	1.0
III-IV	14	14	
Tumor status			
T_{1-2}	17	21	0.372
T_{3-4}	10	6	
Nodal status			
N_0	15	9	0.170
N_{1-2}	12	18	
Adjuvant chemotherapy and/or radiotherapy			
Positive	8	12	0.260
Negative	19	15	

*Derived using Student's *t* test; other *P*s were derived using Fisher's exact.

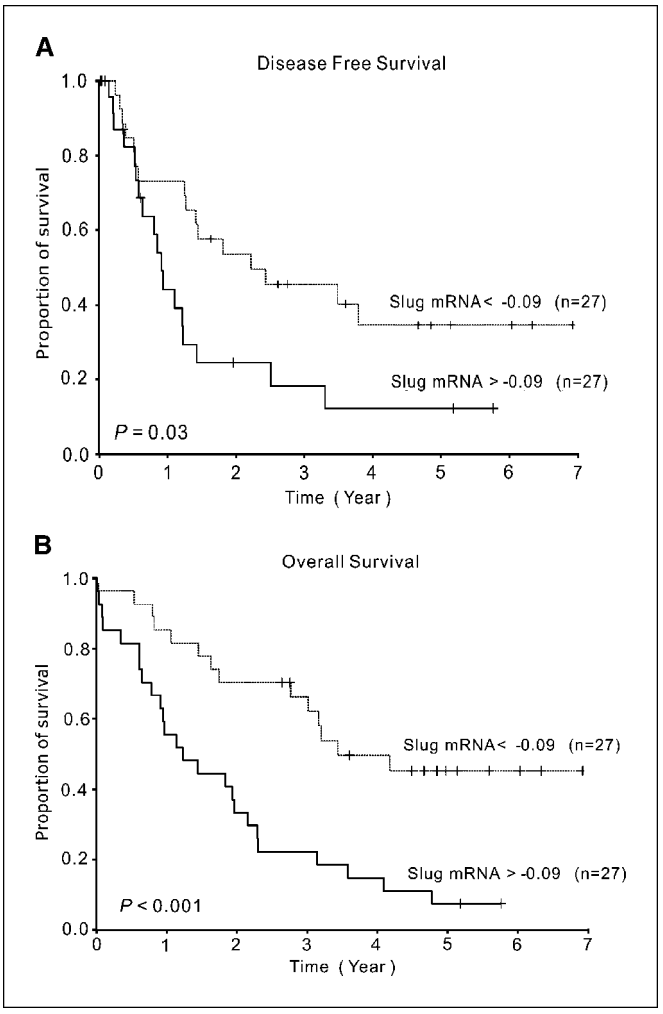

Fig. 1. Kaplan-Meier survival plots of disease-free survival (*A*) and overall survival (*B*) for non – small cell lung cancer patients grouped according to *Slug* mRNA expression. There was a significant difference in disease-free survival ($P = 0.03$) and overall survival ($P < 0.001$) between patients with high and low expression of *Slug* mRNA.

Overexpression of* Slug *promotes* in vitro *carcinoma cells invasion and suppresses E-cadherin expression. *Slug*-transfected CL1-0 clones (Slug4 and Slug6) expressed higher levels of *Slug* mRNA (Fig. 3A) than the control clone (mock) and CL1-0. We used an *in vitro* – reconstituted basement membrane invasion assay to investigate whether *Slug* expression affected the invasive activity of cancer cells. After a 24-hour incubation, we noted a statistically significant increase in the invasive activity of Slug4 ($P = 0.01$) and Slug6 ($P = 0.002$) than the control clone (Fig. 3B). Slug-transfected cells (Slug4 and Slug6) and CL1-5 have similar range of Slug/TBP mRNA ratio (Figs. 2 and 3) and similar *in vitro* invasion abilities (7, 10).

To investigate whether a causal relationship exists between the invasion phenotype and *Slug* expression, Tet-Off *Slug* cDNA-transfected clone (Slug33) and Tet-Off mock transfectant T2 were established. Slug33 displayed elevated levels of *Slug* mRNA compared with doxycycline-treated Slug33 and T2 (doxycycline-treated or untreated; Fig. 3C). We used a Matrigel

invasion assay to determine whether *Slug* expression affects cancer cell invasion. After a 24-hour incubation, a significant increase in invasive potential was noted in Slug33 compared with doxycycline-treated Slug33 and T2 (doxycycline-treated or untreated; $P < 0.01$; Fig. 3D).

Loss of E-cadherin expression is associated with cancer cell invasion. Snail has been firmly established as a repressor of E-cadherin in different types of cancer cells (19). The role of *Slug*, as a potential E-cadherin repressor, has remained uncertain. We used these constitutive and inducible *Slug* expression clones to study the relationship of *Slug* and E-cadherin. *E-cadherin* expression was lost in the overexpressed clones (Slug4 and Slug6; Fig. 3E). In the Tet-Off system, after the addition of doxycycline, the expression of *Slug* in Slug33 clone was suppressed and the expression of *E-cadherin* was desuppressed (Fig. 3F).

To show that suppression of E-cadherin expression is the cause of Slug-induced invasion, we examined whether ectopic expression of E-cadherin could suppressed the invasion ability of Slug-expressed cells. The pCDNA3-*ECad* plasmid was transiently transfected into CL1-5 cells. As shown in Fig. 3G and H, the E-cadherin protein expressed in CL1-5 after transfection and the invasion activity of E-cadherin-transfected CL1-5 was significantly reduced compared with mock control cells ($P < 0.01$). These data show that E-cadherin reexpressed in Slug expression cells could inhibit the invasion ability.

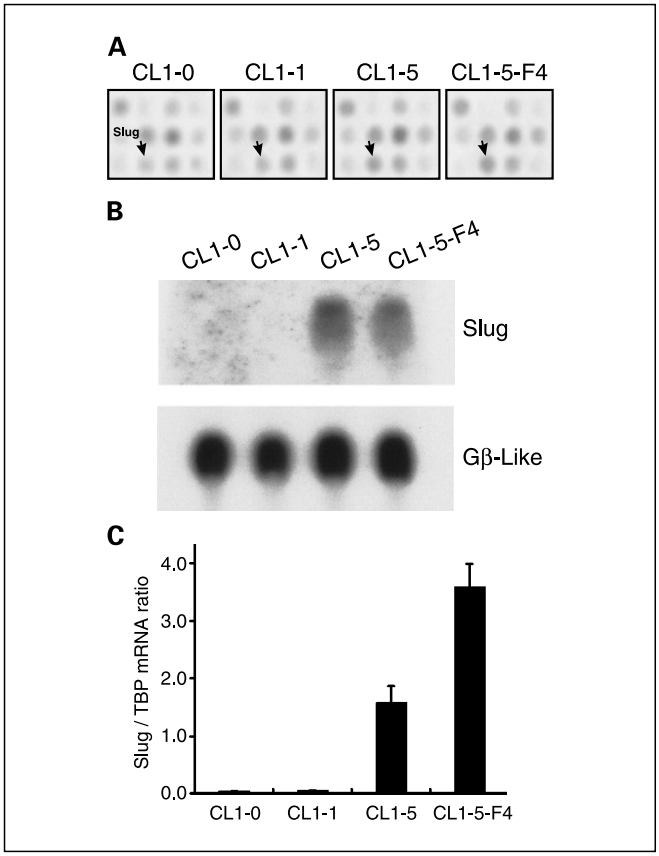

Fig. 2. *Slug* mRNA expression correlated positively with cell line invasiveness. The order of invasive capacities of the cell lines was CL1-0 < CL1-1 < CL1-5 < CL1-5-F4. Microarray (*A*), Northern blot (*B*), and real-time quantitative RT-PCR (*C*) of *Slug* in a series of lung cancer cell lines. *Gβ-like* and *TBP* mRNA were used as an internal control for RNA quantity.

***Expression of* Slug *does not affect cancer cell migration and proliferation* in vitro.** To examine whether the Slug invasion-promoting potential is associated with its promoting on the cell motility, the effects of Slug on the migration capability of cells was analyzed. In the standard scratch wound assay, confluent monolayers of mock, Slug4 and Slug6 cells were scratch wounded using a cell scraper. We noted that there was no significant difference in the migration capability of these cells (data not shown). There was no significant difference in the cell proliferation rates between mock, Slug4, and Slug6 cells as shown by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (data not shown).

Slug *increases the expression and activity of matrix metalloproteinase-2 and* in vivo *murine angiogenesis.* Zymographic analysis was used to assess whether the invasive nature of the Slug overexpression clones correlated with their gelatinase activity. A 66-kDa gelatinase activity could be observed with the Slug4 and Slug6, but low activity was observed for mock control and CL1-0 (Fig. 4A). The ELISA assay of supernatant medium confirmed that the MMP-2 protein amount is higher in Slug4 ($P < 0.001$) and Slug6 ($P = 0.012$) than mock and CL1-0 (Fig. 4B). RT-PCR also showed that *Slug* expression clones (Slug4 and Slug6) had higher expression of *MMP-2* mRNA (Fig. 4C).

Because MMP-2 can regulate angiogenesis, to understand whether Slug expression cells can induce angiogenesis, we did *in vivo* angiogenesis assay. The microvessel count of the tumor of Slug4 (86.7 ± 16.9; $\times 200$ field) was significantly higher than that of the tumor of mock (15.7 ± 6.4; $P < 0.01$, Student's *t* test). The angiogenesis activity of Slug4 increased ~5.5-fold compared with the mock by *in vivo* murine angiogenesis assay (Fig. 4D and E).

Slug *promotes* in vivo *tumor growth.* We next investigated the effect of *Slug* expression on tumorigenicity *in vivo*. Overexpression of *Slug* resulted in a marked increase of the growth of tumors formed by lung cancer cell in SCID mice (Fig. 5A). Our results showed that mock transfectant failed to develop tumors in mice (six of six) 4 weeks after inoculation, whereas Slug4 developed tumors (six of six) larger than 1 cm^3 within 3 weeks. After 7 weeks, the tumor sizes from the mock transfectants were <1 cm^3, whereas the tumor sizes from the Slug4 reached 3.5 cm^3. It is evident that *Slug* expression promotes the tumorigenicity *in vivo*. However, no lung or liver metastasis by microscopic examinations was noted while SCID mice were scarified on week 7. The xenograft tumors were studied with immunohistochemical study and graded by 0 to 2+ as described in Patients and Methods showed increased expression of MMP-2 in the Slug4 clones compared with the mock ($P = 0.038$, Mann-Whitney test). A representative figure was shown in Fig. 5B and C. The expression of E-cadherin in xenograft was scanty in both Slug4 and mock transfectants. We also got the three xenograft tumors of CL1-5 in SCID mice; all three were diffusely stained by anti-MMP-2 antibody (graded as 2+), but all three had no staining by anti-E-cadherin (graded as 0). Representative pictures of expression of MMP-2 and E-cadherin in CL1-5 tumors were shown in Fig. 5D and E.

Discussion

In this study, we showed that *Slug*, a novel invasion-associated gene identified by a genome-wide cDNA microarray

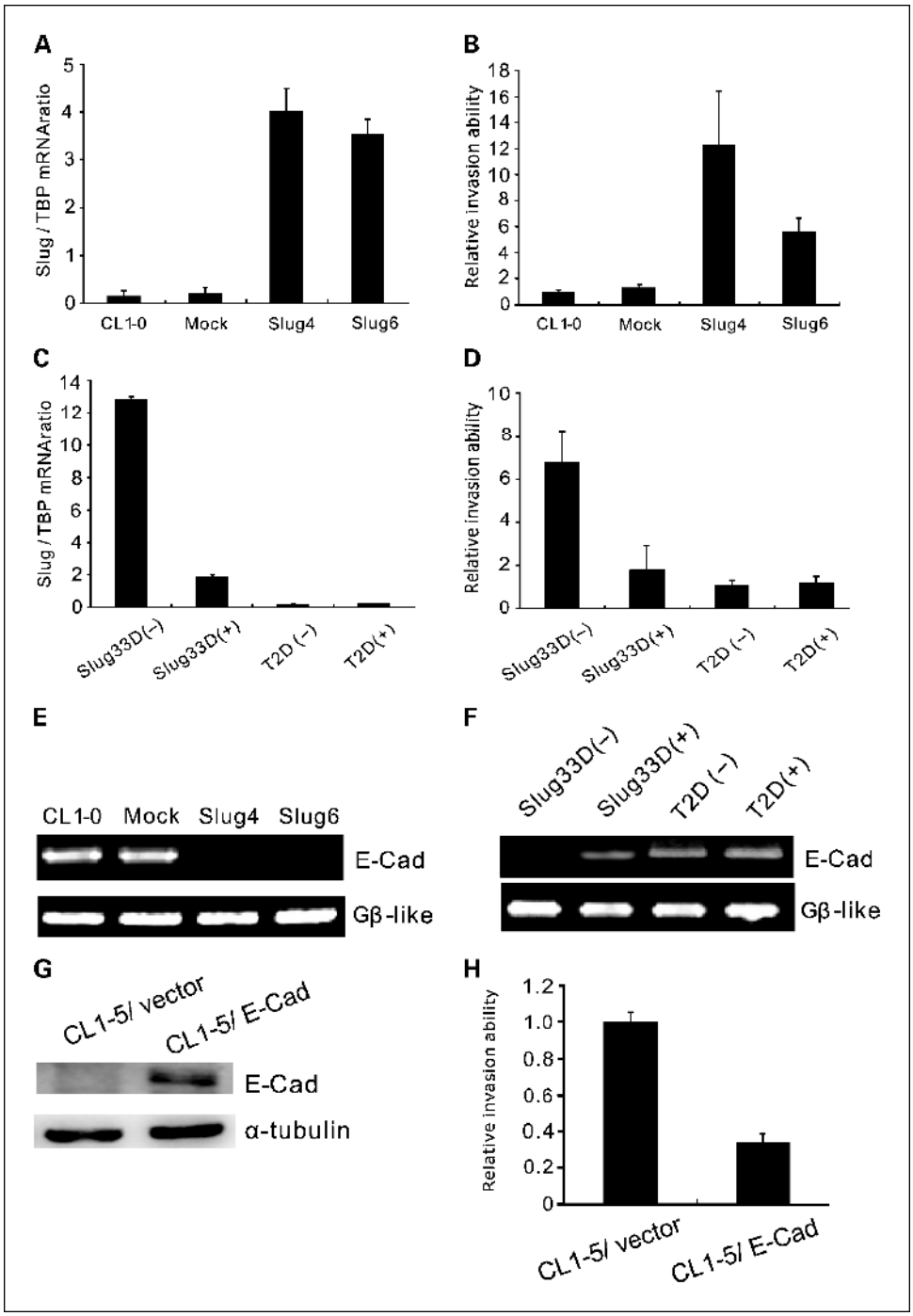

Fig. 3. Overexpression of Slug promotes *in vitro* carcinoma cells invasion and suppresses E-cadherin expression. *A,* quantitative RT-PCR revealed that *Slug*-transfected CL1-0 clones (Slug4 and Slug6) had higher expression of *Slug* than mock transfectant and parental CL1-0. *B,* Slug4 and Slug6 had increased *in vitro* invasion activity compared with the mock transfectant and parental CL1-0. *C,* increased expression of *Slug* in Slug Tet-Off clone (Slug33) was suppressed after addition of 1,000 ng/mL doxycycline. T2, pTRE2 and pTK-Hyg vector transfectant as a control clone; D(+), cells were cultured in the present of doxycycline; D(−), cells were cultured without the present of doxycycline. *D,* increased *in vitro* invasion ability of SlugTet-Off clone (Slug33) was suppressed after the addition of 1,000 ng/mL doxycycline. *E,* RT-PCR showed *E-cadherin* expression decreased in Slug4 and Slug6. *F,* expression of *E-cadherin* was desuppressed when Slug33 was cultured in the presence of doxycycline. *Gβ-like* was used as an internal control for RNA quantity. *G,* pCDNA3-*ECad* plasmid was transiently transfected into CL1-5 cells, which have high Slug expression. The overexpression of E-cadherin in CL1-5 was evaluated by Western blotting. *H,* invasion activity of E-cadherin-transfected CL1-5 was significantly reduced compared with mock-transfected CL1-5 cells.

screening, is indeed an invasion-promoting gene. High *Slug* mRNA expression in lung adenocarcinoma patients can predict postoperative recurrence and shortened survival. Overexpression of *Slug* in lung cancer cells can promote cancer cell invasiveness *in vitro* and angiogenesis and tumor growth *in vivo*. Increase of cancer cell invasion by *Slug* is mediated through the suppression of E-cadherin expression and up-regulation of MMP-2 expression and enzyme activity.

Slug is a member of the Snail superfamily of zinc finger transcription factors (13). The Snail superfamily has been found in *Drosophila* and then in many species, including nonvertebrates, vertebrates, and humans (13). The Snail superfamily in human includes Slug, Snail, Snail-like, Scratch1, and Scratch2 (13). These proteins share an evolutionarily conserved function and are implicated in the generation and migration of mesoderm and neural crest cells in several species (28, 29). EMT is a process that allows epithelial cells to separate from their neighbors and migrate to populate distal regions during embryonic development (30, 31). The EMT confers migratory and invasive properties to epithelial cells and is an essential event during gastrulation movements and neural crest formation but has also been suggested to play a fundamental role during invasion and metastasis of carcinoma cells (31). Snail superfamily members have been implicated in the

occupying a central position in triggering EMT in physiologic and pathologic situations (32).

In recent years, there were studies about how Snail superfamily regulates EMT (33–36). Loss of E-cadherin is found in many cancers and a major phenomenon of EMT. Snail has been firmly established as a repressor of E-cadherin in melanoma cell lines and several epithelial cell lines, such as oral squamous cell carcinoma, hepatocellular carcinoma, breast cancer, and diffuse-type gastric cancer tissue (37, 38). The role of Slug as a potential *E-cadherin* repressor was uncertain. Overexpression of Slug in rat bladder carcinoma cells (NBT-II) was not able to suppress E-cadherin but instead induced desmosome dissociation (29). In addition, a collection of mouse epidermal keratinocyte cell lines did not show any correlation between E-cadherin and Slug expression profiles (19). However, Hajra et al. reported that Slug but not Snail is the repressor of E-cadherin in breast carcinoma cell lines (39). Stable transfection of Slug in Madin-Darby canine kidney cells leads to a full EMT associated with the complete repression of E-cadherin expression (40). In our experiment, we showed overexpression of Slug suppressed *E-cadherin* expression in lung cancer cell lines. These apparent discrepancies in relation to E-cadherin regulation can reflect the specific contribution of different cellular contexts.

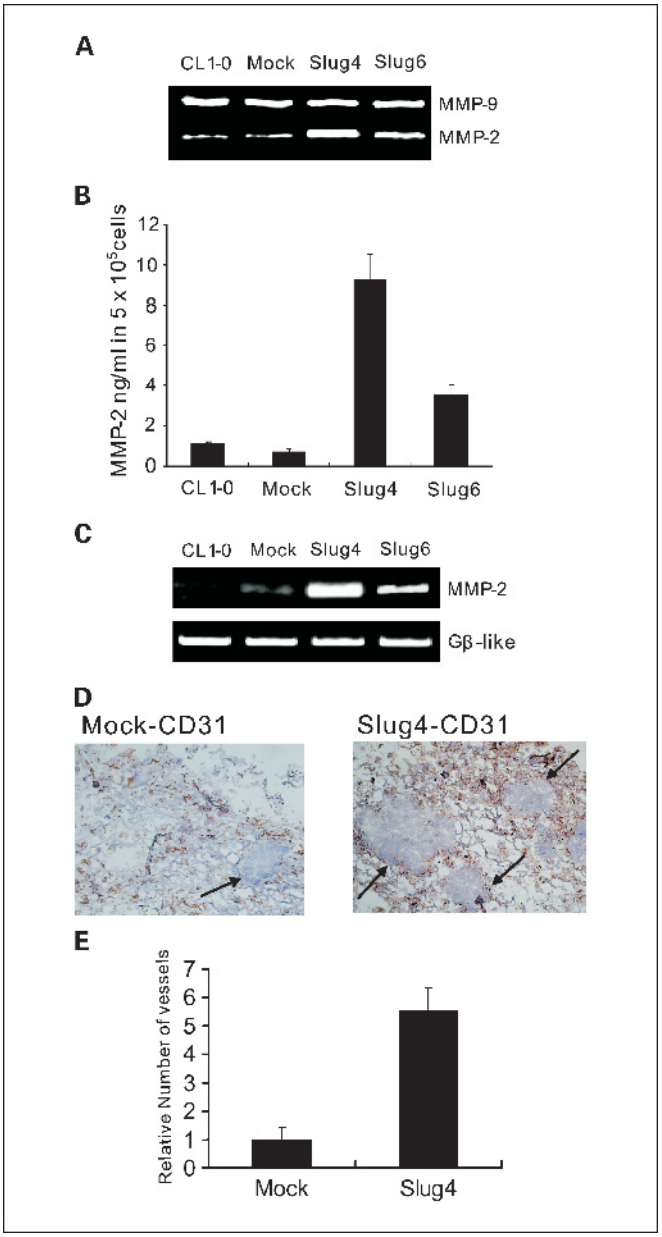

Fig. 4. Slug increased the expression and activity of MMP-2. *A,* zymographic analysis showed increased MMP-2 activity in Slug4 and Slug6. The activity of MMP-9 is similar between these cells. *B,* ELISA assay of supernatant medium confirmed that the MMP-2 protein amount is higher in Slug4 and Slug6. *C,* RT-PCR also showed that Slug4 and Slug6 had higher expression of *MMP-2* mRNA. *D,* representative immunohistochemical staining of the Matrigel plug sections with anti-CD31 antibody shows a significant increase of CD31-positive vessels in plug sections of Slug4 cells compared with mock control cells. Arrows, tumor nests. Original magnification, ×66. *E,* microvessels surrounding the tumor nest were calculated. The angiogenesis activity of Slug4 increased significantly ~5.5-fold compared with the mock control.

Invasion is the early process of metastasis for cancer cells to go through the basement membrane and into the stroma. Invasion is one of the markers of the cellular malignancy and poor prognosis of cancer (4). Previous reports showed Snail-transfected Madin-Darby canine kidney cells have increased Matrigel invasion abilities than control Madin-Darby canine kidney mock clones (18, 40). However, previous reports of Slug transfection studies did not report the invasion analysis. Increase of cancer cell invasion ability in experiment could be due to increase of motility. Our investigation found that the motility of Slug-transfected cells were the same as those of mock-transfected control. This is consistent with the findings that Slug-transfected NBT-II did not increase migration ability compared with parental NBT-II cells (29). However, Bolos et al. reported that the migratory ability of Madin-Darby canine kidney Slug cells in the wound assays was much increased compared with the Madin-Darby canine kidney mock clone (40).

MMPs play an important role in degradation of extracellular matrix, which is an essential step in the cascade of metastasis (41). In the present study, the zymography analysis, ELISA test, and RT-PCR all showed that Slug-transfected cells had increased MMP-2 RNA and protein production and activities. MMP-2 degrades type IV collagen, which is a major component of basement membrane. MMP-2 is a prominent predictor of poor prognosis for patients with various cancers (42). MMP-2 has also been reported to be up-regulated by Snail (43, 44). Snail-transfected A431 (vulval squamous cell carcinoma) showed increased expression of *MMP-2* and the up-regulation of MMP-2 promoter activity (43). Similarly, expression of MMP-2 was up-regulated in Snail-transfected HepG2 (hepatocellular carcinoma cell line; ref. 44). We propose that regulating the extracellular matrix in addition to the loss of cell-cell adhesion is another fundamental role of Slug in promoting cancer invasion.

There were several reports about the expression of *Snail* in human cancer tissue. Cheng et al. did RT-PCR in invasive ductal carcinoma patients and found that the expression of *Snail* increased in those with low E-cadherin (37). Blanco et al. showed that the expression of *Snail* in infiltrative ductal carcinoma patients inversely correlates with the differentiation and positively correlated with lymph node metastasis (38). Sugimachi et al. showed that the *Snail* mRNA correlated with capsular invasion but *Slug* mRNA did not correlate with tumor invasion of hepatocellular carcinoma (45). Martin et al. also reported that neither *Slug* nor *Snail* showed a correlation with tumor-node-metastasis status of breast cancer by quantitative RT-PCR (46). In contrast, Uchikado et al.

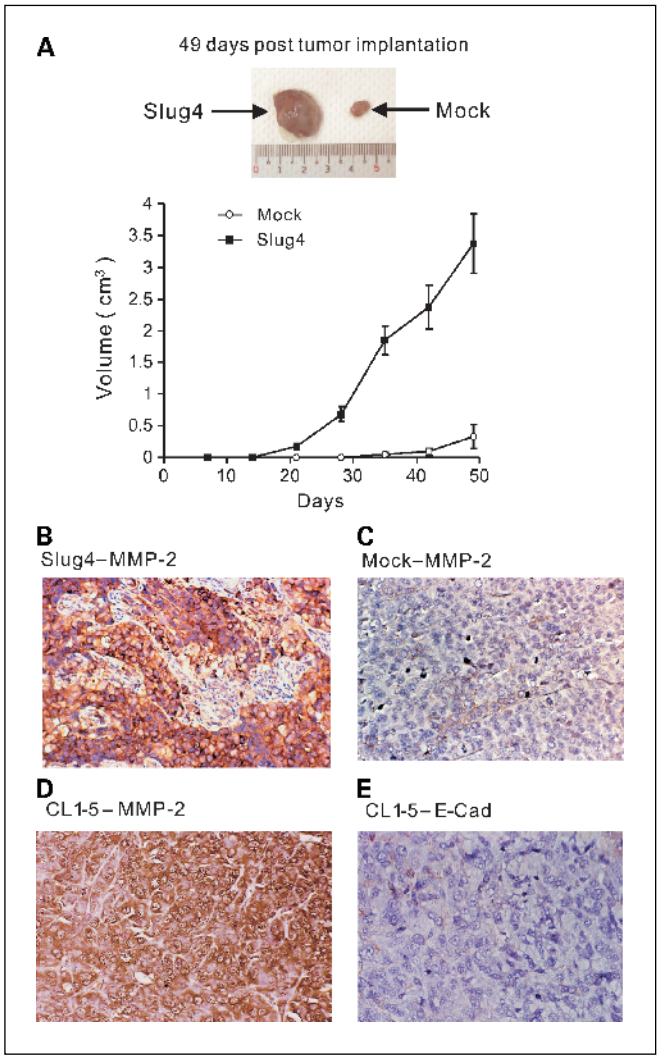

Fig. 5. Slug promoted *in vivo* tumor growth. *A,* tumor development in SCID mice of Slug4 was much faster and larger than the mock transfectant. Bars, SD. Immunohistochemical staining of MMP-2 in representative xenograft tumors show increased expression of MMP-2 in Slug4 (*B*) compared with the mock (*C*). Representative picture of the MMP-2 staining (*D*) and E-cadherin staining (*E*) in xenograft of CL1-5 shows diffused expression of MMP-2 and no repression of E-cadherin. Original magnification, ×66.

reported that Slug was related to depth of tumor invasion, lymph node metastasis, stage, and venous invasion of esophageal cancer by immunohistochemistry study (47). Our study showed that *Slug* mRNA expression promoted cancer cell invasion through suppression of E-cadherin and increase of MMP-2 expression, promoted tumor growth, and was associated with patient survival but was not associated with tumor status, nodal metastasis, or stage. This is quite similar to MMP-2, which promoted cancer cell invasion, promoted tumor growth, and was associated with patient survival but was not associated with lymph node metastasis or cancer stage in lung cancer (48, 49).

Results of *Slug* mRNA expression can predict postoperative recurrence and overall survival of patients with lung adenocarcinoma in this study are consistent with reports of Slug protein expression was associated with adverse survival in patients with esophageal squamous cell carcinoma (47). High *Snail* expression was associated with shorter recurrence-free survival in hepatocellular carcinoma patients (50). There are two points we need to mention. First, cells expressing Slug were resistant to chemotherapy and radiotherapy (16–18). Although there was no statistical difference in the distribution of patients receiving adjuvant therapy between *Slug* mRNA high-expression and low-expression groups, the survival benefit of low-expression group could be partially due to better effect of adjuvant therapy. Second, the cut point of *Slug* mRNA obtained in this study may not be extrapolated to other population samples. The cut point value of *Slug* mRNA should be assessed in sufficiently large prospective study.

The application of large-scale gene expression analysis to cancer studies has made identification of the differentially expressed genes responsible for invasion a practical approach. *Slug* is a metastasis-promoting gene, which has significant value to predict postoperative recurrence and overall survival of patients with lung adenocarcinoma. Disclosing the genes regulated by *Slug* will provide further clues to its biological roles and more generally will contribute to the understanding of the mechanisms underlying the invasion of lung cancer.

Acknowledgments

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