Cancer Research

SLIT2 Attenuation during Lung Cancer Progression Deregulates β-Catenin and E-Cadherin and Associates with Poor Prognosis

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Abstract

Chromosome 4p15.3 is frequently deleted in late-stage lung cancer. We investigated the significance of the SLIT2 gene located in this region to lung cancer progression. SLIT2 encodes an extracellular glycoprotein that can suppress breast cancer by regulating β-catenin. In this study, we examined alterations in the structure or expression of SLIT2, its receptor ROBO1, and β-catenin, along with the AKT/glycogen synthase kinase 3β (GSK3β)/β-transducin repeat-containing protein (βTrCP) pathway in lung cancer cell lines and patients. Low SLIT2 expression correlated with an upward trend of pathological stage and poorer survival in lung cancer patients. Importantly, SLIT2, βTrCP, and β-catenin expression levels predicted postoperative recurrence of lung cancer in patients. Stimulating SLIT2 expression by various methods increased the level of E-cadherin caused by attenuation of its transcriptional repressor SNAI1. Conversely, knocking down SLIT2 expression increased cell migration and reduced cell adhesion through coordinated deregulation of β-catenin and E-cadherin/SNAI1 in the AKT/GSK3β/βTrCP pathway. Our findings indicate that SLIT2 suppresses lung cancer progression, defining it as a novel "theranostic" factor with potential as a therapeutic target and prognostic predictor in lung cancer. *Cancer Res; 70(2); 543–51. ©2010 AACR.*

Introduction

Metastasis is a significant cause of death in lung cancer (1). Therefore, identification of genes and molecular pathways involved in lung cancer metastasis may lead to advances in therapeutics. Our previous data showed a high frequency of loss of heterozygosity at 4p15.3 and 3p12.3, the chromosomal sites of *SLIT2* and *roundabouts 1* (*ROBO1*) genes, in non–small cell lung cancer (NSCLC; refs. 2, 3). In addition, the chromosomal region 4p15.3 was frequently deleted in late-stage, but not early-stage, NSCLC patients, indicating its association with cancer progression (2, 3). SLIT2, a secreted glycoprotein of the SLIT family, encodes the human orthologue of the *Drosophila* Slit2 protein (4). SLIT2 is a ligand of the receptor ROBO1 that transducts intercellular signaling, for example, that of GTPase-activating proteins (5, 6).

The SLIT/ROBO signaling pathway was first found to guide the direction of migration neurons (7). In nonneural cells, SLIT2 was found to inhibit chemotaxis of leukocytes (8, 9) and vascular smooth muscle cells (10) and migration of medulloblastoma cells (11) and breast cancer cells (12). Furthermore, hypermethylation of the *SLIT2* promoter region is frequently found in lung, breast, colorectal, neuroblastoma, renal, and cervical tumors (13–16).

Recently, SLIT2 has been reported to suppress tumor growth by coordinating regulation of the β-catenin and phosphoinositide 3-kinase (PI3K)/AKT pathways in cell and animal models of breast cancer (12). In normal and nonstimulated cells, most β-catenin protein is present in adherens junctions, with very little in cytoplasmic or nuclear fractions as it undergoes rapid turnover by the multiprotein destruction complex containing axis inhibition protein 1 (AXIN1), AXIN2, APC, and glycogen synthase kinase 3β (GSK3β; ref. 17). This complex seems to facilitate the phosphorylation of β-catenin by GSK3β to create a recognition motif for β-transducin repeat-containing protein (βTrCP), an ubiquitin ligase, thus providing for degradation through the ubiquitin-proteasome pathway (18). However, β-catenin degradation is inhibited by GSK3β phosphorylation along the PI3K/AKT pathway (19). In addition, GSK3β promotes SNAI1 phosphorylation and leads to βTrCP-mediated ubiquitination and degradation (20). Nuclear SNAI1 is a potent repressor of E-cadherin expression that regulates the epithelial-mesenchymal transition (EMT; ref. 21).

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To date, the clinical and biological significance of the SLIT2/ROBO1 signaling pathway has not been shown in human lung cancer patients. To investigate the mechanisms involved in SLIT2-mediated tumor progression, we performed a comprehensive molecular analysis of SLIT2/ROBO1, AKT/GSK3β/βTrCP/β-catenin, and SNAI1/E-cadherin alterations in clinical and cellular models to explore the clinical link between these proteins and the mechanisms of SLIT2 antimigration in human NSCLC progression.

Materials and Methods

Clinical characterization of patients. Paired tumor and normal lung tissues were obtained from 92 NSCLC patients recruited at the Taipei Veterans General Hospital between 2002 and 2005 after appropriate institutional review board permission and informed consent from patients were obtained.

Immunohistochemical analysis. Paraffin blocks of tumors were sectioned into 5-μm slices and processed using standard techniques. Antibodies used and their experimental conditions are provided in the Supplementary Data. Staining was scored 3, 2, 1, or 0 if >70%, 36% to 70%, 5% to 35%, or <5%, respectively, of tumor cell nuclei or cytoplasm stained positive for βTrCP. A score of 1 or 0 indicated the presence of little or no SLIT2, ROBO1, and βTrCP. Staining detected at >60% in cell nuclei and cytoplasm indicated β-catenin accumulation.

Immunoprecipitation assay. Catch and Release Reversible Immunoprecipitation System kit (Upstate Chemicon) was used for protein-protein interaction analysis. Detailed procedure for immunoprecipitation with anti–E-cadherin, anti–β-catenin, or normal mouse IgG was described in the Supplementary Data.

Western blot analysis. Cell lysates were collected and immunoblotting was performed for SLIT2, AKT, GSK3β, βTrCP, SNAI1, E-cadherin, and β-catenin under the conditions described in the Supplementary Data.

mRNA expression analysis. Primers for reverse transcription-PCR (RT-PCR) analysis are listed in Supplementary Table S1. Reactions were described in the Supplementary Data. Tumor cells expressing *SLIT2* and *ROBO1* mRNA were normalized with *GAPDH* as the internal control. Those expressing levels <50% that of normal cells were deemed to have an abnormal pattern.

Methylation-specific PCR assay. Primers for the methylation-specific PCR (MSP) assay are listed in Supplementary Table S1. Positive control samples with unmethylated DNA from IMR90 normal lung cell and *Sss*I methyltransferase-treated methylated DNA were included in each PCR set. Hypermethylated genes were defined as those that produced amplified methylation products from the tumor samples.

5-Aza-2′-deoxycytidine treatment of lung cancer cells. CL1-5 human lung cancer cells (with high migration ability) were plated at 10^5 per 100-mm culture dish on the day before treatment. The cells were treated three doubling times with 2 μmol/L 5-aza-2′-deoxycytidine (5-aza-dC) and then harvested for MSP, RT-PCR, Western blot, and migration assays.

Conditioned medium assay. Conditioned medium was collected from low-motility CL1-0 cells with SLIT2 expression. High-motility CL1-5 cells were incubated with a mixture of conditioned medium and fresh medium containing 30% serum, and relative migration ability of treated cells was measured after 48 h. The protein concentration of SLIT2 in condition medium was measured by human SLIT2 ELISA kit (Uscnlife Co.).

Knockdown or ectopically expressed or purified SLIT2 and knockdown AKT analysis. We used pGIPZ lentiviral vector [empty vector without a short hairpin RNA (shRNA) insert]–mediated shRNA-*SLIT2* (Open Biosystem) to generate knockdown clones for the *SLIT2* gene. The small interfering RNA (siRNA)–*AKT* was obtained from Invitrogen Corp. Generation of the pcDNA-*SLIT2* construct is described in the Supplementary Data. CL1-5 and CL1-0 cells (1×10^5) were transfected with 5 μg of shRNA-*SLIT2*, siRNA-*AKT*, or pcDNA-*SLIT2* using ExGen 500 transfection reagent (Fermentas) as recommended by the manufacturer. Human SLIT2 protein was purchased from Abcam Ltd. The cultures were treated with 5 ng/mL SLIT2 protein. After incubation, cells were confirmed by RT-PCR, Western blot, and migration assays.

Transwell migration assay. The Transwell migration assay was performed to determine the migratory ability of shRNA-*SLIT2*–transfected cells and tumor cells treated with 5-aza-dC, purified SLIT2, and ectopically expressed SLIT2. Cells attached to the reverse phase of the membrane were stained and counted under microscope in 10 randomly selected fields.

Wound-healing assay and cell-extracellular matrix adhesion assays. Wound-healing and cell-extracellular matrix adhesion assays were performed for cells treated with 5-aza-dC or ectopically expressed SLIT2 as described in the Supplementary Data.

Statistical analysis. Pearson's χ^2 test was used to compare frequency of protein alterations in NSCLC patients at different disease stages. Overall survival curves and disease-free survival curves were calculated according to the Kaplan-Meier method, and comparison was performed using the log-rank test. $P \leq 0.05$ was considered statistically significant. Statistical Package for the Social Sciences version 13.0 (SPSS, Inc.) was used for all statistical analyses.

Results

Correlation of altered SLIT2/ROBO1 and βTrCP/β-catenin pathways with cancer progression and poor prognosis in NSCLC patients. To examine the role of the SLIT2/ROBO1 and βTrCP/β-catenin pathways in cancer progression of NSCLC, immunohistochemical analysis of SLIT2 and ROBO1 was performed on samples from 92 NSCLC patients. Due to sample availability, βTrCP and β-catenin analysis was performed on samples from 74 patients (Fig. 1*A*). Immunohistochemical data indicated that 41%, 21%, and 19% of tumors showed an absence or low expression of SLIT2, ROBO1,

and βTrCP protein, respectively, whereas 60% of tumors showed β-catenin accumulation. Low expression of SLIT2 and βTrCP was associated with an upward trend of pathologic stage in lung cancer samples (P = 0.003–0.017; Fig. 1*B*). In stratification analyses, low expression of SLIT2 and βTrCP, singly or together, was correlated in late-stage patients with β-catenin accumulation (P = 0.002–0.014; Supplementary Fig. S1). In addition, low expression of SLIT2 or ROBO1 was found in late-stage patients with β-catenin accumulation (P = 0.025; Supplementary Fig. S1).

To define the prognostic effects of altered SLIT2, βTrCP, and β-catenin expression in lung cancer patients, survival curves were estimated using the Kaplan-Meier method. Lower levels of SLIT2 expression were associated with overall

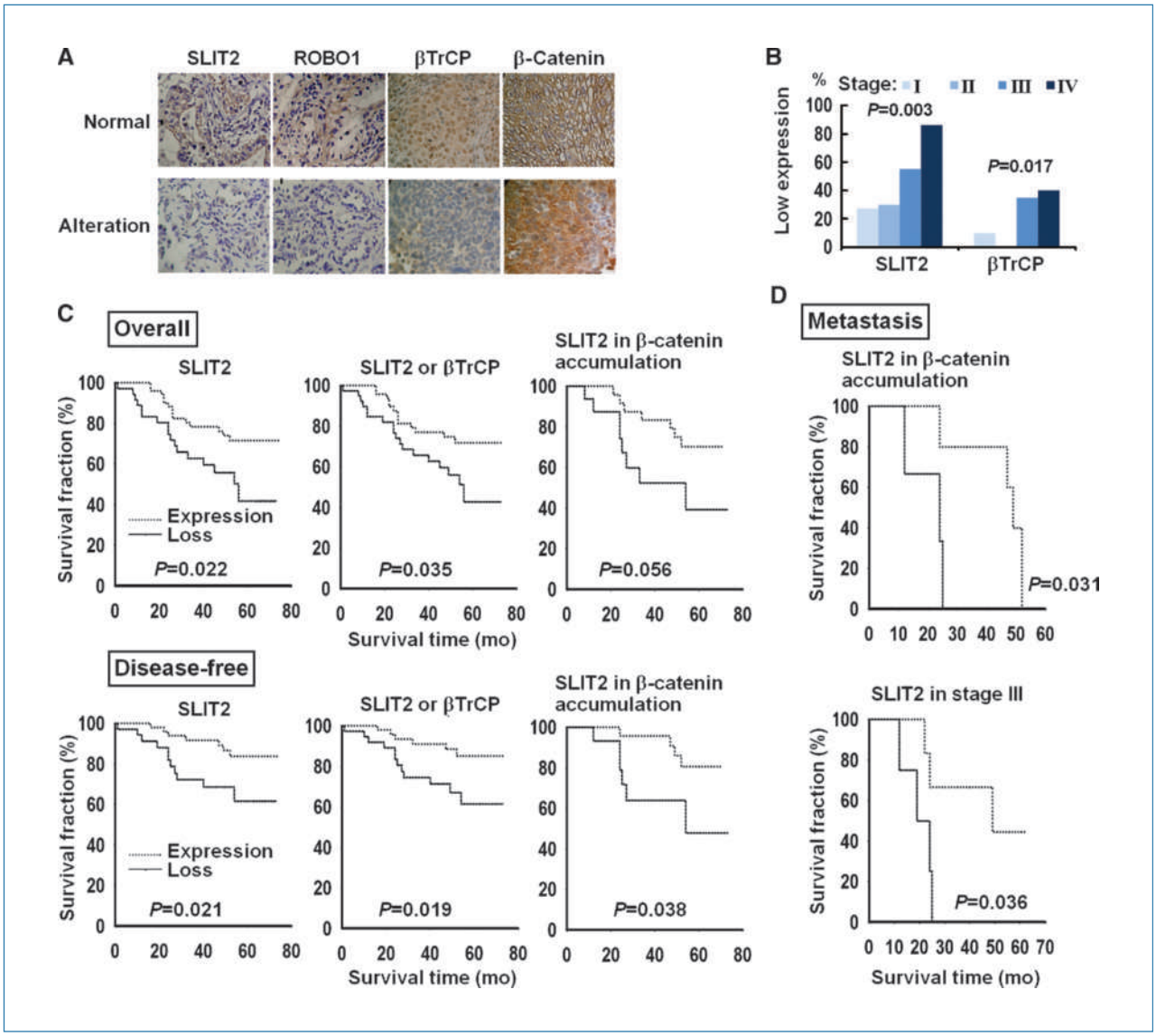

Figure 1. Protein expression and survival analyses of SLIT2, ROBO1, βTrCP, and β-catenin in NSCLC patients. *A,* representative immunohistochemical analysis of SLIT2, ROBO1, βTrCP, and β-catenin proteins in formalin-fixed, paraffin-embedded tissues. *Top,* normal expression pattern of SLIT2, ROBO1, βTrCP, and β-catenin proteins; *bottom,* cells from patients with altered expression of these proteins. Evaluation criteria are described in Materials and Methods. Original magnification, ×200. *B,* correlation of altered SLIT2 and βTrCP expression with stage progression. *P* values for each comparison are given above the bars. *C,* Kaplan-Meier survival curves with respect to low protein expression of SLIT2 and/or βTrCP and β-catenin accumulation. *Expression,* expression of SLIT2 in mRNA or protein; *Loss,* loss of expression of SLIT2 in mRNA and protein. The graphs show the overall survival and disease-free survival of patients by SLIT2 expression (*left*), of patients with alteration of either SLIT2 or βTrCP expression (*middle*), and of patients with β-catenin accumulation with alteration of either SLIT2 or βTrCP expression (*right*). *P* value for each analysis is as indicated. *D,* in those having tumor with metachronous metastasis, disease-free survival is shown for patients with SLIT2 expression, adjusted for β-catenin accumulation and being at tumor stage III. *P* value for each analysis is as indicated.

poor prognosis and disease-free survival of NSCLC patients (P = 0.022 and P = 0.021, respectively; Fig. 1*C*). In addition, less SLIT2 or βTrCP expression correlated with worse prognosis in overall and disease-free survival (P = 0.035 and P = 0.019, respectively; Fig. 1*C*). In NSCLC patients with β-catenin accumulation, lower SLIT2 expression was associated with poorer prognosis of disease-free survival (P = 0.038; Fig. 1*C*). Importantly, patients with metachronous metastasis and lower expression of SLIT2 had significantly shorter survival times with β-catenin accumulation (P = 0.031) and at tumor stage III (P = 0.036; Fig. 1*D*).

mRNA expression and promoter hypermethylation of the SLIT2 and ROBO1 genes in NSCLC patients. To verify whether epigenetic alterations are involved in low SLIT2 and ROBO1 protein expression, we carried out mRNA expression and DNA methylation assays of *SLIT2* and *ROBO1* genes in this cohort of 92 NSCLC patients (Fig. 2*A*; Supplementary Fig. S2*A*). Semiquantitative RT-PCR analysis showed that decreased or absent *SLIT2* and *ROBO1* transcripts were found in 45% and 11%, respectively, of tumor tissues compared with normal tissues. MSP assay of tumor cells from 92 patients showed that 52% and 31% of tumors exhibited promoter hypermethylation of *SLIT2* and *ROBO1* genes, respectively. We subsequently sought to correlate mRNA expression and promoter methylation status (Fig. 2*B*; Supplementary Fig. S2*B*). Low mRNA expression was significantly associated with promoter hypermethylation (SLIT2, P = 0.008; ROBO1, P = 0.022). Aberrant protein expression was significantly associated with low mRNA expression (SLIT2, P < 0.001; ROBO1, P < 0.001).

mRNA and protein of SLIT2 are reactivated by 5-aza-dC treatment. A lung metastasis cell model, which included a

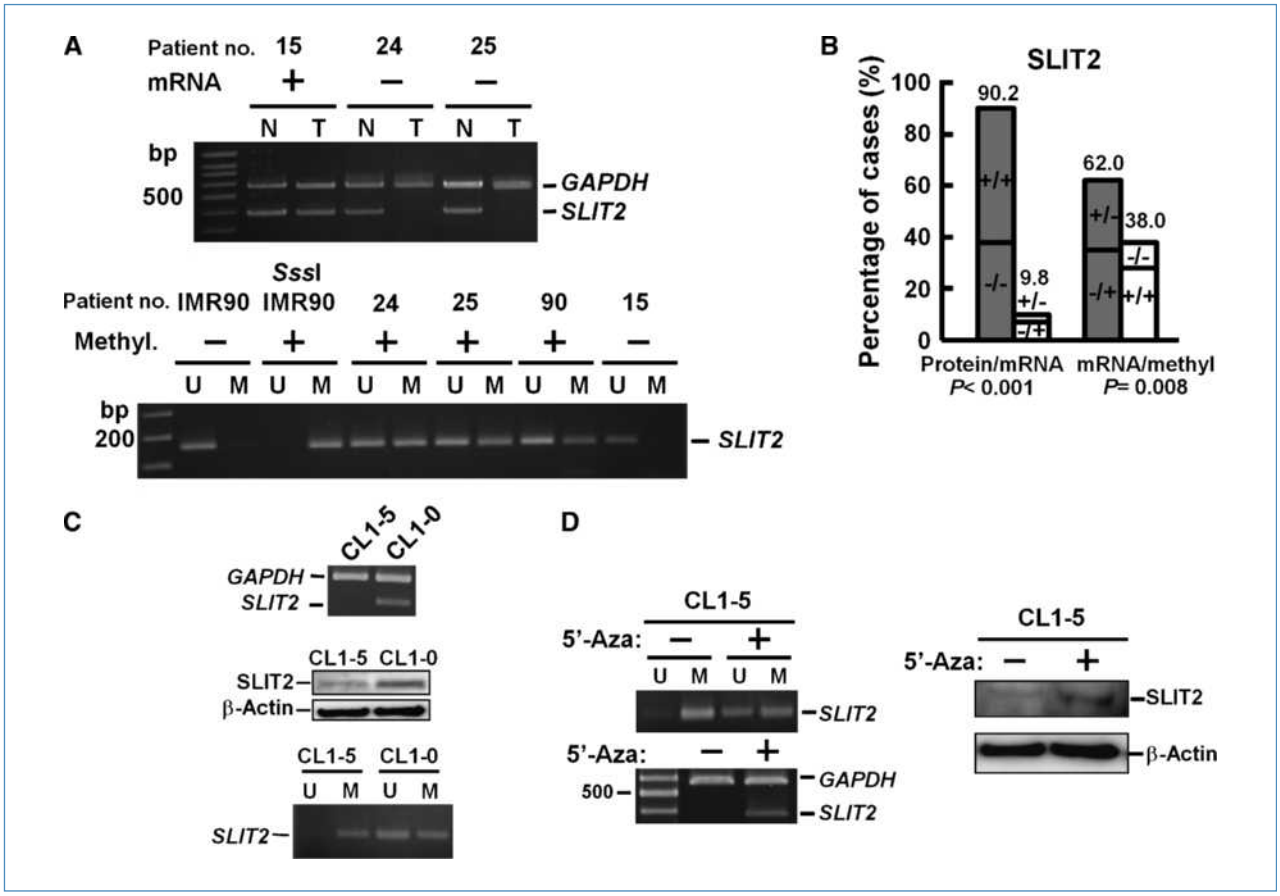

Figure 2. Epigenetic analyses of *SLIT2* in NSCLC patients and cell lines. *A,* mRNA (by RT-PCR; *top*) and promoter methylation (by MSP; *bottom*) analyses of *SLIT2* in representative NSCLC patients with (+) and without (–) methylation expression. *N,* normal lung tissue; *T,* tumor lung tissue. The primer sets used for amplification. *M,* methylated genes. IMR90 is a normal lung cell line used as an unmethylated control and *Sss*I-treated IMR90 as a methylated control. *B,* concordance analysis between protein, mRNA, and promoter methylation for the *SLIT2* gene. *Y axis,* percent of cases; *X axis,* type of comparison. Positive (+) and negative (–) expression status and methylated (+) and unmethylated (–) promoter status are noted. The percentage in the concordant group (*gray columns*) and nonconcordant group (*white columns*) is indicated above. *P* values are shown at the bottom. *C,* two lung cancer cell lines, CL1-5 and CL1-0, were selected as our model cells. The highly metastatic CL1-5 was methylated at the promoter region of the *SLIT2* gene with and without *SLIT2* mRNA and protein expression. *D,* effects of 5-aza-dC (*5′-Aza*) treatment on CL1-5 cells. MSP analysis showed demethylation of *SLIT2* gene as indicated by amplification of U reaction in CL1-5 cells after 5-aza-dC treatment. *SLIT2* mRNA and protein were restored after treatment with 5-aza-dC.

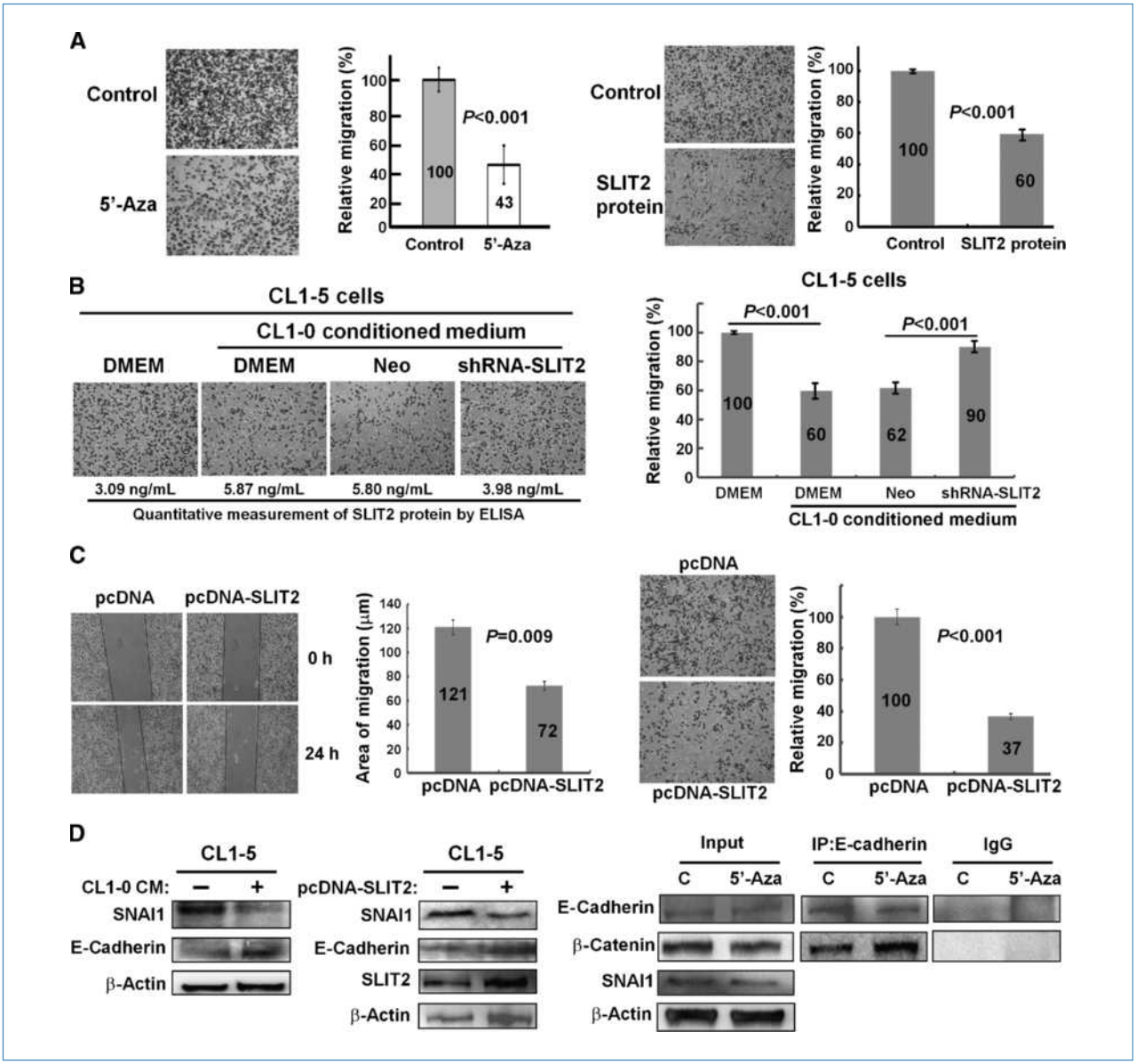

Figure 3. Reexpression of SLIT2 suppresses its relation to the E-cadherin/β-catenin pathway and the migration of CL1-5 lung cancer cells. *A,* high migration potential was seen in CL1-5 cells before 5-aza-dC treatment by Transwell migration assay. *Left,* migration ability decreased dramatically after treatment with 5-aza-dC; *right,* migration was inhibited in CL1-5 cells with the addition of purified SLIT2 protein (5 ng/mL) by Transwell migration assay. *B,* migration was inhibited in CL1-5 cells after adding conditioned medium (*DMEM*) from CL1-0 cells, but migration suppression was attenuated with the addition of medium from CL1-0 cells with *SLIT2* knockdown construct (*shRNA-SLIT2*). The concentration of SLIT2 in the medium was measured by ELISA and is indicated below. *C, left,* wound-healing assay showed that ectopically expressed SLIT2 (*pcDNA-SLIT2*) suppressed cell migration; *right,* ectopic expression of SLIT2 in CL1-5 cells suppressed cell migration by Transwell assay. Photographic views of migration assay are shown. Relative migration was calculated in four independent experiments. *D,* Western blot for E-cadherin in CL1-5 cells treated with or without SLIT2 conditioned medium (*CM*; *left*) and ectopically expressed SLIT2 (*pcDNA-SLIT2*; *middle*). *Right,* immunoprecipitation assay using anti–E-cadherin antibody shows increased interaction of β-catenin and E-cadherin with 5-aza-dC treatment.

low-migration lung cancer cell line CL1-0 and its derivative cell line CL1-5 with a high motility (22), was used for the following experiments. CL1-5 cells showed a hypermethylated *SLIT2* promoter and low expression of SLIT2 mRNA and protein, whereas the parental CL1-0 cells showed normal methylation and expression levels of SLIT2 (Fig. 2*C*).

To determine whether *SLIT2* promoter methylation was the predominant mechanism causing loss of SLIT2 gene expression, the CL1-5 cells were treated with the demethylating agent 5-aza-dC. As shown in Fig. 2*D*, treatment with 5-aza-dC successfully demethylated the promoter region of *SLIT2* gene and restored SLIT2 mRNA and protein expressions.

Migration suppression of CL1-5 cells treated with 5-aza-dC, purified SLIT2 protein, conditioned medium from CL1-0 cells, or ectopically expressed SLIT2. To verify the role of SLIT2 reactivation in lung cancer migration, we performed Transwell migration experiments in CL1-5 cells with and without 5-aza-dC. We found a significant decrease in migration of CL1-5 cells with 5-aza-dC treatment compared with untreated control cells ($P < 0.001$; Fig. 3*A, left*). Because SLIT2 is primarily a secreted glycoprotein, we sought to analyze the effect of purified SLIT2 protein or conditioned medium harvested from CL1-0 cells (which presumably contain SLIT2 protein) or CL1-5 cells by Transwell assay. The data showed that addition of purified SLIT2 protein (5 ng/mL) or CL1-0 conditioned medium (5.8 ng/mL) decreased the migration capacity of CL1-5 cells to 60% that of control cells ($P < 0.001$; Fig. 3*A* and *B, right*); this effect was not observed when conditioned medium from shRNA-*SLIT2* knockdown CL1-0 cells was added ($P < 0.001$; Fig. 3*B, right*). In addition, ectopically expressed SLIT2 in CL1-5 cells resulted in a significant decrease in migration capacity compared with the empty vector control cells, as determined by wound-healing and Transwell assays ($P = 0.009$ and $P < 0.001$, respectively; Fig. 3*C*).

Migration suppression of SLIT2 in relation to β-catenin/E-cadherin pathway. The β-catenin/E-cadherin complex is one of the major regulators of EMT (18, 21). To validate the mechanism of SLIT2-mediated migration suppression, E-cadherin and SNAI1 expression was examined in CL1-5 cells with and without conditioned medium from CL1-0 cells and ectopically expressed SLIT2. Conditioned medium from CL1-0 with SLIT2 increased E-cadherin and decreased SNAI1 expression in CL1-5 cells (Fig. 3*D, left*). Similarly, ectopically expressed SLIT2 in CL1-5 cells resulted in enhanced E-cadherin expression and reduced SNAI1 expression compared with the empty vector control cells (Fig. 3*D, middle*). To examine the extent to which the β-catenin/E-cadherin complex was associated with SLIT2-mediated migration suppression, CL1-5 cells with and without 5-aza-dC were immunoprecipitated with E-cadherin antibody and then Western blotted for β-catenin and E-cadherin proteins. CL1-5 cells with 5-aza-dC showed both in-

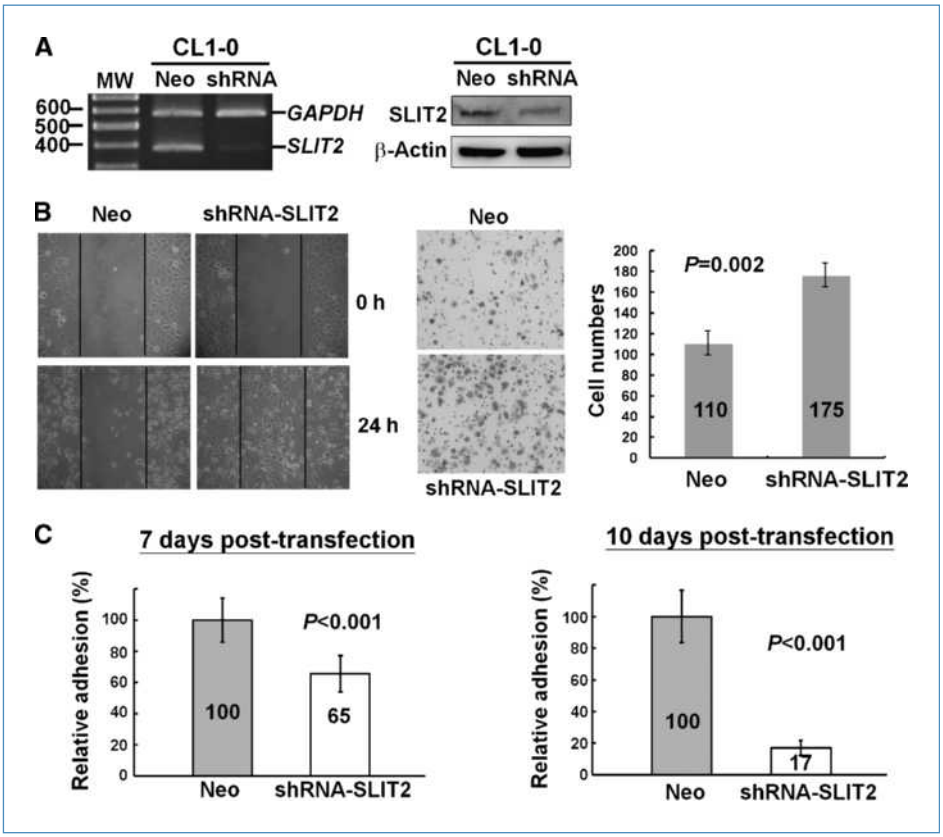

Figure 4. Inverse correlation of SLIT2 expression and cell motility as seen using *SLIT2* knockdown in CL1-0 lung cancer cells. *A,* SLIT2 mRNA (*left*) and protein (*right*) expression were decreased in CL1-0 cells with *SLIT2* knockdown. Expression of the housekeeping genes *GAPDH* and *β-actin* was used as an internal control. *B, left,* wound-healing assay showed that shRNA-*SLIT2*–transfected cells invaded the wound 24 h later, but no apparent invasion occurred in the control cells; *right,* similarly, high migration potential was seen in the shRNA-*SLIT2*–transfected CL1-0 cells by Transwell migration assay. Histogram shows a significant increase in migration capacity in shRNA-*SLIT2*–transfected cells in four independent experiments. *P* value is as indicated. *C,* adhesion assay. Adhesion potential was seen in CL1-0 cells before transfecting with shRNA-*SLIT2* construct by adhesion assay. Adhesion ability was reduced to 65% and 17% in CL1-0 cells with *SLIT2* knockdown at 7 and 10 d after transfection, respectively, compared with neo vector controls.

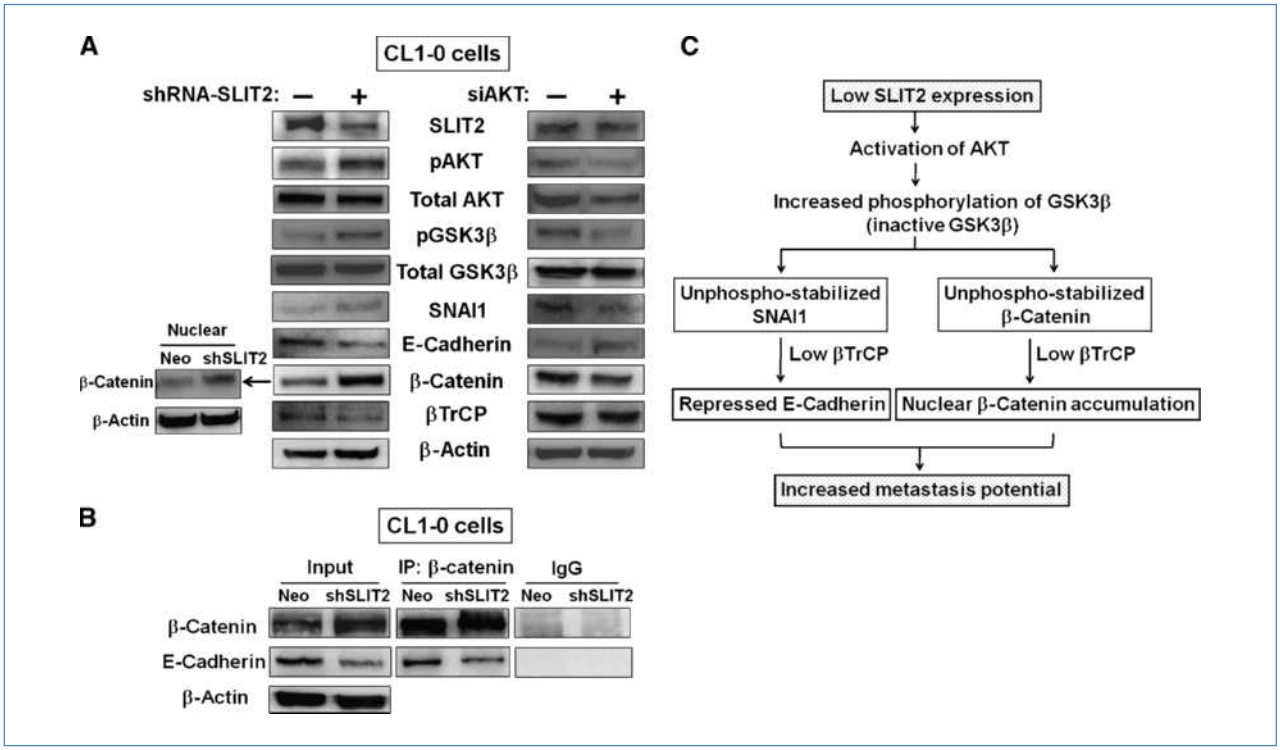

Figure 5. Activation of the AKT pathway in response to low SLIT2 expression coincides with increased cell motility and decreased cell adhesion. *A,* Western blots of SLIT2, AKT pathway proteins, SNAI1, E-cadherin, and β-catenin in *SLIT2* and *AKT* knockdown CL1-0 cells compared with untreated CL1-0 cells. *Left,* nuclear β-catenin levels increased in cells with *SLIT2* knockdown. Expression of the housekeeping gene *β-actin* was used as an internal control. *Right,* SLIT2 is upstream of AKT signaling, as confirmed by *AKT* knockdown. *B,* immunoprecipitation assay using the anti–β-cadherin antibody shows decreased interaction of β-catenin with E-cadherin in *SLIT2* knockdown CL1-0 cells compared with control cells. *C,* model of inactive SLIT2 leading to increased metastasis in lung cancer cells. Low expression of SLIT2 may activate the AKT pathway and result in phosphorylation of GSK3β, an inactive form that triggers the βTrCP-mediated degradation of β-catenin and SNAI1 proteins. The accumulated β-catenin, perhaps in conjunction with low E-cadherin levels caused by SNAI1 transcriptional repression, is important in the loss of cell adhesion and increased metastatic potential in lung cancer patients.

creased β-catenin/E-cadherin association (Fig. 3*D, right*) and decreased cell migration (Fig. 3*A*).

Knockdown of SLIT2 increases cell motility in lung cancer cell line. To further confirm the reciprocal relationship between SLIT2 expression and cell motility in lung cancer, we used lentiviral vector–mediated shRNA technology to generate knockdown of the *SLIT2* gene in the lung cancer cell line CL1-0. By RT-PCR and Western blot assays, *SLIT2* knockdown CL1-0 cells showed lower *SLIT2* mRNA and protein expression compared with neo vector control (Fig. 4*A*). Next, we tested for cell motility by wound-healing and Transwell assays. As shown in Fig. 4*B*, *SLIT2* knockdown CL1-0 cells showed significantly greater migration capacity compared with untreated CL1-0 cells. In addition, we studied the effect of *SLIT2* knockdown on adhesive property. Adhesion was reduced to 65% in CL1-0 cells at 7 days after transfection ($P < 0.001$), and to 17% at 10 days after transfection in the *SLIT2* knockdown construct ($P < 0.001$), compared with neo vector controls (Fig. 4*C*).

Knockdown of SLIT2 alters β-catenin/E-cadherin levels by AKT/GSK3β/βTrCP signaling in lung cancer cell line. To examine the relationship between SLIT2 and β-catenin/E-cadherin protein levels and their correlation with the AKT pathway in lung cancer cells, *SLIT2* and *AKT* knockdown CL1-0 cells were examined for SLIT2, β-catenin, E-cadherin, and AKT signaling protein expression. Western blot analysis showed that shRNA-*SLIT2* knockdown was accompanied by a low level of E-cadherin and high levels of SNAI1 and β-catenin expression in CL1-0 cells. In addition, β-catenin expression dramatically increased in the nuclear fraction. We further evaluated the expression of AKT/GSK3β/βTrCP destruction signal proteins in *SLIT2* knockdown CL1-0 cells. Phospho-AKT and phospho-GSK3β increased and βTrCP decreased (Fig. 5*A, left gel*). Conversely, *AKT* knockout induced E-cadherin expression and decreased expression levels of SNAI1, phospho-GSK3β, and β-catenin in CL1-0 cells. However, SLIT2 expression was similar in *AKT* knockdown and control CL1-0 cells, suggesting that SLIT2 operates upstream of AKT signaling (Fig. 5*A, right gel*).

To test whether the association between β-catenin and E-cadherin is mediated by SLIT2, immunoprecipitation with β-catenin antibody and then Western blotting with β-catenin and E-cadherin were performed for *SLIT2* knockdown CL1-0 cells and vector control cells. The data indicated that *SLIT2* knockdown decreased the interaction of β-catenin and E-cadherin (Fig. 5*B*), confirming that loss of SLIT2 can increase cell migration and decrease β-catenin/E-cadherin complex formation.

Discussion

In the present study, we provide the first compelling evidence that SLIT2 is a suppressor of NSCLC progression. The SLIT2/ROBO1 pathway is important in controlling NSCLC cell migration by its coordinated regulation of β-catenin and E-cadherin levels in the AKT/GSK3β/βTrCP pathway. Low expression of SLIT2 correlates with β-catenin accumulation, low level of E-cadherin, late-stage disease, and poor survival, suggesting that SLIT2 can serve as a prognostic biomarker of NSCLC metastasis.

Previous studies have indicated that *SLIT2* may be a tumor suppressor gene, but its antimigration property has not yet been reported in human lung cancers. Our clinical data show for the first time that loss of SLIT2 correlates with stage progression and predicted postoperative cancer recurrence in NSCLC patients (Fig. 1). In addition, cell motility was reduced with reactivated SLIT2 expression and growth in SLIT2-containing medium and ectopically expressed SLIT2, but *SLIT2* knockdown increased cell motility in a lung cancer cell model (Figs. 2–4). Consistent with our data, low expression of *SLIT2* gene has been found in invasive cervical cancer (16) and esophageal squamous cell carcinomas (23). SLIT2 expression inhibits invasion of medulloblastoma cells (11) and migration of breast cancer cells (12). Recently, Kim and colleagues (23) showed that, in nude mice, SLIT2-transfected fibrosarcoma HT1080 cells formed significantly fewer pulmonary metastatic nodules than either parental or control vector–transfected cells. These data add support to our clinical and cellular model data, indicating that SLIT2 suppresses the migration of tumor cells *in vivo*.

β-Catenin is important in E-cadherin–mediated cell-cell adhesion (24). Therefore, we examined the expression of E-cadherin in highly metastatic CL1-5 lung cancer cells treated or not with conditioned medium from low-metastasis CL1-0 cells with SLIT2 expression or ectopically expressed SLIT2. Indeed, our data showed that conditioned medium from CL1-0 cells with SLIT2 and ectopically expressed SLIT2 increased E-cadherin expression in CL1-5 cells and decreased motility. In addition, E-cadherin enhanced the association with β-catenin in SLIT2 reactivated with 5-aza-dC (Fig. 3). Conversely, SLIT2 knockdown decreased β-catenin/E-cadherin association and increased cell motility (Fig. 5). Together, our results indicated that the β-catenin/E-cadherin complex, the major regulator of EMT, is involved in SLIT2-mediated migration suppression.

Overexpression of SLIT2 was associated with decreased β-catenin expression resulting from the increased AKT/GSK3β/βTrCP signal in a lung cancer cell line, confirming the data from the breast cancer cell line (12). We further examined *SLIT2* and *AKT* knockdown in low-motility CL1-0 cells with SLIT2 and AKT expression. Loss of SLIT2 expression increased cell migration and reduced cell adhesion in CL1-0 cells. In shRNA-*SLIT2* knockout CL1-0 cells, we found high levels of β-catenin, SNAI1, phospho-AKT, and phospho-GSK3β and low levels of βTrCP and E-cadherin. In addition, SLIT2 expression was similar in *AKT* knockdown and neo vector control CL1-0 cells, confirming that low levels of SLIT2 enhanced the AKT pathway to inactivate GSK3β for β-catenin and SNAI1 degradation in a lung cancer model (Fig. 5). Our data provide the first evidence that SLIT2 regulates E-cadherin expression through GSK3β and SNAI1. SNAI1 has been shown, in concert with β-catenin accumulation, to prompt EMT by repressing E-cadherin expression (21). Accumulated stable β-catenin in the cytoplasm translocates into the nucleus and then activates expression of target genes such as *MMP7* that regulate cell migration (25, 26). In addition, reduced expression of E-cadherin disrupts the β-catenin/E-cadherin complex and results in decreased cell adhesion, differentiation, and metastasis (27). Finally, low βTrCP expression may result from the low levels of its substrates (28), such as phospho–β-catenin and SNAI1, in *SLIT2* knockdown cells. Our previous study showed that knockdown of the *βTrCP* gene increases β-catenin expression in lung cancer cells (29). Relevantly, He and colleagues (30) found that knockdown of βTrCP accelerates cell invasion of lung cancer cells.

Regulation of cell growth and apoptosis has been suggested as the mechanism of SLIT2-mediated suppression of tumor growth (14, 15). To examine the effect of SLIT2 on cell growth in our lung cancer model, cell counting assay was performed on cells treated with 5-aza-dC, those with SLIT2 overexpression, and *SLIT2* knockdown. No significant difference was observed between the manipulated lung cancer cells and control cells (Supplementary Fig. S3*A*). In addition, we found no apparent induction of apoptosis and no change in cell cycle distribution between CL1-5 cells with or without 5-aza-dC, *SLIT2* knockdown CL1-0 cells, and control cells by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay and flow cytometry (Supplementary Fig. S3*B* and *C*). Consistent with our data, Kim and colleagues (23) found no significant difference in cell growth between the anchorage-dependent parental and SLIT2-transfected fibrosarcoma and squamous cell carcinoma. Their experiments did not show a significant difference in apoptosis between SLIT2-transfected and control cells in culture. However, apoptosis regulation has been suggested as a SLIT2-mediated effect on colorectal tumor cells (15). We speculate that the effect of SLIT2 may differ by cell type.

Our clinical and cell model findings reveal a new mechanism resulting from activation of the AKT/GSK3β/βTrCP pathway in response to low SLIT2 expression in NSCLC: β-catenin accumulation and E-cadherin reduction (Fig. 5*C*). The causal role of SLIT2 and AKT downstream signaling was confirmed by *SLIT2* and *AKT* knockdown approaches. These findings provide a new dimension to our understanding of SLIT2-mediated tumor migration and also open up a new line of potential cancer therapeutics to attenuate lung cancer metastasis. Targeting SLIT2 activity in tumor cells is an attractive goal in cancer therapy because SLIT2 is a secreted glycoprotein that may be lost in highly metastatic cancer cells. Transducible peptides, such as SLIT2 peptide, represent a promising new technology for efficient delivery of designer therapeutic molecules into cells. The rare alteration of the SLIT2 receptor ROBO1 in lung cancer increases the potential effectiveness of SLIT2 peptide therapy. In addition, methylation of *SLIT2* promoter results in increased

lung cancer metastasis. Therefore, reactivating the SLIT2 function by reversing epigenetic inactivation may also represent a novel therapeutic opportunity to attenuate human lung cancer. More functional analyses correlating inactive SLIT2/ROBO1 signaling and constitutive activation of the CDC42/WASP/ARP pathway (5) to delineate the role of SLIT2 in cell biology and cancer metastasis are needed.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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