L1 Cell Adhesion Molecule Promotes Tumorigenicity and Metastatic Potential in Non–Small Cell Lung Cancer

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Abstract

Purpose: Non–small cell lung cancer (NSCLC) is a highly metastatic cancer with limited treatment options, thus requiring development of novel targeted therapies. Our group previously identified L1 cell adhesion molecule (L1CAM) expression as a member of a prognostic multigene expression signature for NSCLC patients. However, there is little information on the biologic function of L1CAM in lung cancer cells. This study investigates the functional and prognostic role of L1CAM in NSCLC.

Experimental Design: Cox proportional hazards regression analysis was done on four independent published mRNA expression datasets of primary NSCLCs. L1CAM expression was suppressed by shRNA–mediated silencing in human NSCLC cell lines. Effects were assessed by examining in vitro migration and invasion, in vivo tumorigenicity in mice, and metastatic potential using an orthotopic xenograft rat model of lung cancer.

Results: L1CAM is an independent prognostic marker in resected NSCLC patients, with overexpression strongly associated with worse prognosis. L1CAM downregulation significantly decreased cell motility and invasiveness in lung cancer cells and reduced tumor formation and growth in mice. Cells with L1CAM downregulation were deficient in constitutive extracellular signal–regulated kinase (Erk) activation. Orthotopic studies showed that L1CAM suppression in highly metastatic lung cancer cells significantly decreases spread to distant organs, including bone and kidney.

Conclusion: L1CAM is a novel prometastatic gene in NSCLC, and its downregulation may effectively suppress NSCLC tumor growth and metastasis. Targeted inhibition of L1CAM may be a novel therapy for NSCLC. Clin Cancer Res; 18(7); 1–11. ©2012 AACR.

Introduction

Lung cancer is one of the most lethal cancers with an estimated 1.3 million deaths per year worldwide (1). Non–small cell lung cancer (NSCLC) comprises approximately 85% of all lung cancers. Despite recent identification of many oncogenes and tumor suppressor genes important for the development and treatment of NSCLC, overall 5-year survival remains poor at 15% (2–6). Furthermore, although early-stage patients potentially are curable by surgery, approximately 30% to 60% recur postoperatively and die from metastatic disease (7). Factors contributing to early progression and metastasis in NSCLC remain largely unknown. The identification of new pathways responsible for tumor progression is critical to the development of both novel prognostic markers and therapies for NSCLC.

Metastasis is associated with profound changes in cellular properties, including the disruption of cell–cell and cell–matrix adhesions and the acquisition of migratory abilities. Deregulated expression of members of all major cell adhesion molecule families [integrins, selectins, cadherins, and the immunoglobulin (Ig) superfamily] is frequently reported in human cancers (8). One such molecule, L1 cell adhesion molecule (L1CAM), is aberrantly expressed in several cancer types, including ovarian (9), melanoma (10), breast (11), pancreatic (12), and colon cancers (13). L1CAM is a type I membrane glycoprotein of the Ig-like superfamily, consisting of 6 Ig-like domains and 5 fibronectin-type III repeats, followed by a single-pass transmembrane region and a highly conserved cytoplasmic tail (14). The extracellular region of L1CAM binds to a diverse range of proteins that include L1CAMs themselves, other cell surface proteins such as integrins and axonin1, and extracellular matrix proteins such as laminin and neurocan.
Translational Relevance

Thirty to 60% of early-stage non–small cell lung cancer (NSCLC) patients still develop metastatic recurrence after complete surgical resection. Although adjuvant chemotherapy has been shown to modestly increase the survival of these patients, novel targeted therapies are urgently needed to improve adjuvant treatment. This study shows that in NSCLC with high L1CAM expression, tumor growth and systemic metastasis are inhibited by L1CAM downregulation in subcutaneous and orthotopic models, respectively. We also confirm that L1CAM is a poor prognostic marker for patients with NSCLC and plays a significant role in promoting invasiveness and motility of NSCLC cells. The results of our study highlight targeting of L1CAM as a potentially important novel therapy for NSCLC patients.

(15, 16). L1CAM function has been most extensively studied in the nervous system, where it is known to orchestrate morphogenetic events such as neuron–neuron adhesion, axon guidance, neurite outgrowth, neurite fasciculation, and myelination (15, 17). Consistent with these functions, mutations in human L1CAM confer a variety of neurologic disorders and L1CAM knockout mice show defective corticospinal tract development (17–19).

The function of L1CAM in lung cancer growth and metastasis is unknown. In this study, we characterize tumor growth and metastatic potential of L1CAM in subcutaneous mice and orthotopic nude rat models of lung cancer, respectively. We show that L1CAM plays a significant role in stimulating cell migration, invasiveness, and systemic metastasis in vivo, thereby establishing L1CAM as a novel bona fide therapeutic target for NSCLC.

Materials and Methods

Prognostic significance of L1CAM in NSCLC patients

The prognostic value of L1CAM expression was assessed in silico using published NSCLC microarray data from 4 patient cohorts (Supplementary Table S1). The JBR.10 was a phase III randomized trial of adjuvant chemotherapy (cisplatin and vinorelbine) versus observation in stage IB-II patients (20, 21). For prognostic analysis, only patients in observation arm were analyzed. The National Cancer Institute Directors’ Challenge Consortium (DCC) cohort included 442 adenocarcinoma patients from 4 North American cancer centers. Excluding patients from the JBR.10 cohort contributed to this DCC study and patients who received adjuvant chemo/radiotherapy, expression data from the 311 patients were used for prognostic analysis (22). The University of Michigan cohort consisted of 129 stage I–III squamous cell carcinomas (23). Gene expression analyses from the above 3 cohorts were done using the Affymetrix U133A microarray. A final cohort included 172 surgically resected NSCLC patients from the Netherlands Cancer Institute (NLCI). Their gene expression profiling was conducted by Agilent 44k microarray (24). Recurrence-free survival was used as the survival end point for JBR.10 and NLCI data sets, and 5-year survival was used for DCC and Michigan data sets, in which recurrence-free survival was not available. The association of the expression of L1CAM and survival was evaluated using Cox proportional hazards regression in SAS v9.2 (SAS Institute) with gene expression as a continuous variable.

On the basis of UCSC genome browser GRCh37/hg19 assembly, L1CAM mapped to 2 Affymetrix probe sets (for both U133A and U133 plus 2). Probe set 204584_at covers all 3 transcripts whereas 204585_s_at covers only 2 transcripts; thus, probe set 204584_at was used to represent L1CAM expression. In the Agilent 44K, C_MaP1_0_ID41179 or A_24_P207995 represents transcript variant 1, which is the longest transcript, and this was used to represent the expression of L1CAM.

Quantitative real-time PCR validation

Quantitative real-time PCR (qRT-PCR) assays were done on 156 RNA samples from the University Health Network (UHN) NSCLC patient cohort as previously described (25). mRNA copy number for each sample was quantified in HT7900 fast real-time PCR System (Applied Biosystems) and calculated by standard curve methodology. The copy number was adjusted by the geometric mean of 4 housekeeping genes (TBP, BAT1, B2M, and ACTB) and log-transformed (25). L1CAM primers targeting the 204584_at probe set region were used: HsL1CAM-fw: 5′-AAAGGAAA-GATTGGTTCCTCCAG-3′; HsL1CAM-rev: 5′-AGTAGAC-CAAGGCACAGGATCATACAG-3′.

Generation of L1CAM short hairpin RNA gene silencing and overexpression

L1CAM gene expression was stably knocked down using short hairpin RNAs (shRNA) against different regions of human L1CAM. All shRNA sequences were obtained from RNAi Consortium (TRC2). The target sequences for shL1CAM5 and shL1CAM2 were 5′-GCCAATGGCTAATGCTAGTT-3′ and 5′-GCTAACCCTAGGTTAAGGAT-3′, respectively. Human full-length L1CAM cDNA plasmid, pH1A-pcDNA3, was obtained commercially (Addgene) and was subcloned into our modified Gateway recombination lentiviral expression vector, plKO.puro.DEST (26), containing a puromycin selection marker. Transduced cells were selected by 1 μg/mL puromycin. For controls, nonspecific shRNA against GFP (shGFP) was used for cell lines with knockdowns, whereas empty vector construct was used for overexpressed cell lines.

Generation of mutant L1CAM sequence resistant to shL1CAM5

L1CAM cDNA insensitive to shL1CAM.5 was produced by insertion of 3 point mutations (C1359G, C1362T, and C1365T) at the shRNA target region by QuikChange II XL Site-directed Mutagenesis Kit (Stratagene), according to the
and 5% CO₂. Multiporous (8-μm pore size) polycarbonate membranes (Falcon). The upper chambers of the membrane were coated with 25 μg Matrigel or 7.5 μg of collagen IV, and the lower chambers were filled with media supplemented with 10% FBS as a chemoattractant. After 48 hours, cells were fixed with 0.5% glutaraldehyde and stained with 0.5% crystal violet reagent. Cotton swabs were used to remove the cells from the upper surface of the membrane, leaving the migratory cells on the underside. The membrane was removed and mounted onto glass slides and scanned. Quantitative image analysis is described in the Supplementary Materials.

Subcutaneous tumorigenicity assay

One million cells were injected subcutaneously in the left shoulder region of 6- to 8-week-old male SCID mice (n = 8 per cell line). Mice were examined every 2 days, and tumor length and width were measured using calipers. Tumor volume was calculated using the following formula (length × width²)/6. Mice were sacrificed once the humane endpoint (approximately 1.5 cm diameter) was reached. At sacrifice, portions of tumors were snap-frozen and stored in liquid nitrogen or were fixed in 10% buffered formalin for routine histopathologic processing.

Orthotopic model to evaluate metastatic potential

Two groups of rats (n = 13 per group) were randomly assigned for orthotopic implantation of H460SM-shL1CAM or H460SM-shGFP control cells. Details for endobronchial tumor cell implantation in nude rats was reported previously (28). Before tumor implantation, animals received 5-Gy whole-body γ-radiation using the Gamma Cell 40 Exactor (Nordion International Inc.; 500 rad of whole-body γ radiation from a 127Cs source and at 120 rad/min). Cultured tumor cells were harvested by trypsinization and adjusted to a final concentration of 1.0 × 10⁶ cells per 50 mL. Rats were anesthetized by intramuscular injection of ketamine/xylazine (110 and 12 mg/kg; CDMV, Inc.) and endobronchially implanted with 1.0 × 10⁶ cells using a 20-gauge, 1.88-inch long Teflon catheter. The cell suspension was injected through a catheter passed into the distal bronchus of the right caudal lobe through a small tracheotomy incision. After withdrawal of the catheter, the tracheotomy was repaired with a 6-0 Proline suture (Ethicon Inc.), and the incision was closed with sterile wound clips.

Assessment of metastatic potential

When any animal from any group succumbed to the disease or showed terminal signs and symptoms, a single animal from the other group was killed, allowing a direct comparison of both groups at the same time point for tumor-associated end points. Animals that died in less than 21 days after implantation were considered postanesthetic tumor-associated end points. Animals that died in less than 21 days after implantation were considered postanesthetic or surgical related complications and were excluded from the final comparative analysis. All tissues were fixed in 10% buffered formalin, serially sectioned, and stained with hematoxylin and eosin for microscopic examination. The primary tumor weights, which reflect the tumor burden, were recorded and portions of the tumor were snap-frozen and stored in liquid nitrogen. Metastases were assessed macroscopically and microscopically in internal organs including lung, kidney, brain, chest wall, and bone as well as mediastinal lymph nodes. Organs or tissues were counted as either positive or negative for metastasis. No assessment of number or extent of metastasis was made.

Statistics

All statistical analyses were carried out with SAS v9.2 (SAS Institute) and GraphPad Prism 5.0. Statistical significance was determined using 2-tailed Student’s t-test or mixed-model ANOVA for invasion and migration assays. Difference in

Animals and cell lines

Four-week-old male nude (CR:NIH-RNU) rats (Charles River, Inc.) were acclimatized for 1 to 2 weeks before entering study protocols. Rats were housed in sterilized cages and fed autoclaved food and water ad libitum. Severe combine immunodeficient (SCID) mice were bred on site and obtained from the Ontario Cancer Institute (OCI; Toronto, ON) animal facility. All manipulations were carried out under sterile conditions in a laminar flow hood, in accordance with procedures approved by the OCI Animal Care Committee.

The NCI-H460, NCI-H125, and NCI-H1264 cell lines were obtained from the American Type Culture Collection and verified by short-tandem repeat (STR) polymorphism analyses. H460SM is a clonal variant of NCI-H460 selected for its high metastatic ability (27). All cells were cultured in RPMI media supplemented with 10% FBS (Hyclone Europe, Ltd.) and antibiotics. All cells were cultivated at 37°C and 5% CO₂.

Western blot analysis

Whole-cell extracts were applied to SDS-PAGE and transferred to polyvinylidene fluoride membranes. Primary antibodies were used were anti-L1CAM (UJ127; Novus Biologicals), β-actin, pErk1/2, Erk1/2, pAkt, Akt, pSrc, Src (Cell Signaling), FAK, and pFAK (BD Transduction Laboratories). Visualization used horseradish peroxidase–linked anti-rabbit and anti-mouse secondary antibodies (Cell Signaling) and ECL-Plus blotting substrate detection kit on a Typhoon phospho- and fluorescent imaging system 9400 (GE Healthcare).

Invasion and migration assay

A total of 5 × 10⁵ cells were seeded onto 24-well transwell cell culture plates (BD Biosciences) fitted with multiporous (8-μm pore size) polycarbonate membranes (Falcon). The following primers were designed (nucleotide mismatch is underlined):

L1mut_fw: 5'-GGGCTTGGCCTGGAATATCAGTTATATTACGTGGTCC-3';
L1mut_rev: 5'-GGACCACTAACATACGTTGGCAGCAAGAGCCC-3'

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L1mut_rev: 5'-GGACCACTAACATACGTTGGCAGCAAGAGCCC-3'
tumor growth rates of xenografts was tested using mixed-model ANOVA. \( \chi^2 \) and Fisher exact test was used for metastasis experiments. All biologic experiments are presented as mean \( \pm \) SEM. Tests that produced \( P \leq 0.05 \) were considered to be significant.

Results

Prognostic impact of L1CAM in NSCLC patients

The 4 published microarray studies included a total of 674 early-stage surgically resected NSCLC patients who had not received adjuvant chemotherapy or radiotherapy. Univariate survival analyses revealed that high L1CAM expression was significantly associated with poorer survival in JBR.10 NSCLC cohort (HR = 2.41, 95% CI = 1.51–3.82, \( P = 0.0002 \)), University of Michigan squamous cell carcinoma cohort (HR = 2.59, 95% CI = 1.51–4.47, \( P = 0.0006 \)), DCC adenocarcinoma cohort (HR = 1.49, 95% CI = 1.03–2.17, \( P = 0.036 \)), and marginally associated in the NLCI NSCLC cohort (HR = 2.53, 95% CI = 0.92–6.95, \( P = 0.072 \); Table 1). Multivariate Cox regression analysis adjusted for tumor histologic subtype, stage, age, and sex showed that L1CAM expression was an independent prognostic factor for the JBR.10 and Michigan patient cohorts and marginally in DCC and NLCI (Table 1 and Supplementary Table S2). Using a qRT-PCR on mRNA of 156 NSCLC UHN patients, we confirmed further the prognostic value of L1CAM expression (multivariate HR = 1.32, 95% CI = 1.00–1.75, \( P = 0.053 \); Table 1).

Establishment of stable L1CAM suppressed and overexpressed NSCLC cell lines

We screened L1CAM protein expression by Western blot analysis in 12 different NSCLC cell lines and an immortalized but nontumorigenic human bronchial epithelial cell line (HBE135). L1CAM expression levels varied significantly among NSCLC cell lines and no L1CAM was detected in HBE135 cells (Fig. 1A). Due to their high endogenous levels of L1CAM protein, the NCI-H460 large-cell carcinoma, NCI-H125 adenosquamous carcinoma, and H460SM large-cell carcinoma cell lines were used for subsequent knockdown experiments, whereas NCI-H1264 adenocarcinoma cells, which contain low levels of endogenous L1CAM protein, were chosen for overexpression studies. Targeting of L1CAM expression in NCI-H460 cells using 2 different shRNAs resulted in reduction of L1CAM protein levels by 76% and 70%, respectively (Fig. 2B). Stable transduction of shL1CAM.5 in NCI-H125 and H460SM cells reduced L1CAM protein levels by 82% and 71%, respectively. Likewise, transduction of shL1CAM.2 targeting a different region of L1CAM decreased protein levels in NCI-H125 and H460SM cells by 92% and 77%, respectively (Fig. 2B). To study the consequences of overexpression, we stably transduced NCI-H1264 cells with an L1CAM expression construct, which resulted in a 10-fold increase in L1CAM levels (Fig. 2B). Stable integration and expression of L1CAM or shRNAs in all cell lines were monitored for more than 10 passages and did not alter the morphology of the cells, which remained epithelial in appearance.

The role of L1CAM in migration, invasion, and Erk pathway activation

Given the known involvement of L1CAM in axon guidance, we first examined whether modulating endogenous L1CAM expression affected cell migration in NSCLC. In our time-lapse wound healing assay, knockdown of L1CAM expression in H125 and H460SM cells significantly

Table 1. Prognostic impact of L1CAM in NSCLC patients

<table>
<thead>
<tr>
<th>Cohort</th>
<th>Tumor type</th>
<th>Platform</th>
<th>No. of patients</th>
<th>( HR^a ) (95% CI)</th>
<th>( P )</th>
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<tr>
<td>JBR.10 (21)</td>
<td>NSCLC</td>
<td>U133A</td>
<td>62</td>
<td>2.41 (1.51–3.82)</td>
<td>0.0002</td>
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<td>DCC (22)</td>
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<td>Michigan (23)</td>
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<td>U133A</td>
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<td>2.59 (1.51–4.47)</td>
<td>0.0006</td>
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<tr>
<td>NLCI (24)</td>
<td>NSCLC</td>
<td>44K</td>
<td>172</td>
<td>2.53 (0.92–6.95)</td>
<td>0.072</td>
</tr>
<tr>
<td>UHN (25)</td>
<td>NSCLC</td>
<td>qRT-PCR</td>
<td>156</td>
<td>1.30 (1.00–1.69)</td>
<td>0.050</td>
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</table>

Univariate survival analysis

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<th>Cohort</th>
<th>Tumor type</th>
<th>Platform</th>
<th>No. of patients</th>
<th>( HR^a ) (95% CI)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>JBR.10 (21)</td>
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<tr>
<td>DCC (22)</td>
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<td>311</td>
<td>1.40 (0.96–2.03)</td>
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<td>Michigan (23)</td>
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<td>U133A</td>
<td>129</td>
<td>2.73 (1.54–4.84)</td>
<td>0.006</td>
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<tr>
<td>NLCI (24)</td>
<td>NSCLC</td>
<td>44K</td>
<td>172</td>
<td>2.53 (0.92–6.95)</td>
<td>0.072</td>
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<tr>
<td>UHN (25)</td>
<td>NSCLC</td>
<td>qRT-PCR</td>
<td>156</td>
<td>1.32 (1.00–1.75)</td>
<td>0.053</td>
</tr>
</tbody>
</table>

Multivariate survival analysis

Abbreviations: DCC, Director’s Challenge Consortium adenocarcinoma; NLCI, Netherlands Cancer Institute; UHN, University Health Network; NSCLC, non–small cell lung cancer; U133A, Affymetrix U133A chip; 44K, Agilent 44K gene expression array; qRT-PCR, real-time quantitative PCR, ADC, adenocarcinoma; SQCC, squamous cell carcinoma; CI, confidence interval.

\( ^a \) HR compares the overall survival of the high-risk patient group to that of the low-risk group.
decreased the rate of lateral migration into a wound introduced in a confluent monolayer of cells compared with controls (P < 0.0001; Fig. 2A). The transmigration ability of these same cells was evaluated on collagen IV–coated transwell filters. Knockdown of L1CAM in cell lines harboring different shRNA sequences resulted in a 70% to 80% decrease in the number of migrating cells compared with controls (Fig. 2B). Cell proliferation did not differ between L1CAM knockdown and control cell lines in 10% serum, indicating that altered migratory potential was not a result of changes in cell growth (Supplementary Fig. S1A–B).

To evaluate the role of L1CAM in invasion, we examined the effect of L1CAM downregulation on the ability of cells to invade Matrigel, a reconstituted basement membrane. The invasiveness of H460 and H460SM cells with reduced L1CAM expression was only 26% and 32% of that of control cells, respectively (Fig. 2C). We also conducted this assay with H1264 cells overexpressing L1CAM to determine whether ectopic L1CAM levels could promote cell invasion. Overexpression of L1CAM resulted in a 3-fold increase in cell invasion of Matrigel, confirming that L1CAM plays a pivotal role in regulating both the migration and invasiveness of lung cancer cell lines.

As previous studies have suggested that the effects of L1CAM on cell migration may be mediated via direct integrin interaction (29), we assayed integrin-mediated signaling pathways by Western blot analysis. Levels of total focal adhesion kinase (FAK) and activated FAK (autophosphorylation site on tyrosine-391) were determined by harvesting cells after serum starvation for 24 hours. No differences were detected in the levels of FAK autophosphorylation between control and L1CAM knockdown cells (Supplementary Fig. S1C). In addition, we did not observe any differences in the level of activated Src (phosphorysine-416), another downstream target of integrin signaling.

We next examined whether L1CAM interacts with the extracellular signal–regulated kinase (Erk) pathway in NSCLC cells, as previously reported in fibroblast and melanoma cells (30). After incubation in serum-free media for 24 hours, we observed no phosphorylated Erk1/2 (pErk1/2) in cells with L1CAM knockdown, compared with low levels of constitutively activated Erk1/2 in control cells (Fig. 2D, compare lane 1 and 2; Supplementary Fig. S1C). When these cells were stimulated with 10% serum, Erk1/2 was activated similarly in both control and L1CAM knockdown cells, suggesting that sustained L1CAM-dependent Erk1/2 activation requires co-operation with one or more serum growth factors. Erk1/2 activity declined to undetectable levels by 24 hours postserum treatment in cells with L1CAM knockdown, whereas control cells expressing high levels of endogenous L1CAM sustained pErk1/2 expression (Fig. 2D, lanes 9 and 10; Fig. 2E). Although Erk1/2 activation in both control and L1CAM knockdown cells peaked at 10 minutes following stimulation, pErk1/2 levels began to decline more rapidly after 120 minutes in cells with L1CAM knockdown compared with the control.

To confirm that the effect of L1CAM knockdown on Erk1/2 activation is not an off-target or viral effect, we transduced H125-shL1CAM cells with lentivirus-expressing mutant L1CAM cDNA, which harbors silent mutations in shL1CAM targeting sequences, and is therefore resistant to shL1CAM-induced L1CAM degradation. Restoring L1CAM in L1CAM knockdown H125 cells reversed the loss of pErk1/2 levels after 24 hours of serum stimulation (Fig. 2F). These results suggested that L1CAM function in NSCLC may be mediated via the Erk pathway.

L1CAM Suppression in NSCLC Cells Reduces in Vivo Tumorigenicity

Given the biologic implications of our in vitro results for the progression of NSCLC, we next examined whether...
Figure 2. Role of L1CAM on migration, invasion, and Erk pathway in NSCLC cell lines. A, time-lapse wound-healing assay was used to monitor cell front migration for 45 hours in H460SM and H125 cells. The rate of wound area traveled by cells with L1CAM knockdown was compared with controls. B, cells were seeded onto transwell membranes coated with collagen IV and the number of migrated cells were counted. C, Matrigel-coated transwell assays were used to examine cell invasion of three different cell lines. A–C, results shown represent more than 3 biologic replicates (\(P < 0.01\); \(P < 0.001\); \(P < 0.0001\)). D, after 24 hours serum starvation, H125-shL1CAM and control cells were stimulated with 10% serum for indicated times before harvesting to assess Erk activation. Whole-cell lysates were subjected to Western blot analysis with anti-phospho-Erk1/2 and anti-Erk1/2 as a loading control. Densitometric quantification of relative amounts of pErk1/2. One of 3 experiments with comparable results is shown. E, H125 and H460 cells were synchronized by serum starvation and incubated in 10% serum for 24 hours before harvesting at 80% confluence to assess Erk activation. F, restoring L1CAM in L1CAM knockdown H125 cells (shL1CAM.5 + L1mut) reversed the loss of pErk levels when cells were maintained in 10% serum for 24 hours after serum starvation. The levels of \(\beta\)-actin were used as internal protein loading controls.
L1CAM contributes to tumorigenicity in vivo. We subcutaneously injected 4 independent clones of H460-shL1CAM cell lines, representing 2 different shRNA sequences, into SCID mice (Fig. 3A). L1CAM downregulation in H460 cells significantly reduced tumor growth rate when compared with controls (\( P < 0.0001 \); Fig. 3A), and these results were verified in an adenosquamous carcinoma cell line model, H125 (\( P < 0.0001 \); Fig. 3B, Supplementary Fig. S3C). Moreover, the effect could be rescued by restoring L1CAM levels using shRNA-resistant L1CAM cDNA (\( P < 0.0001 \); Fig. 3C). To confirm that both H460-shL1CAM and H125-shL1CAM cells continued to express lower L1CAM levels in vivo compared with controls, we carried out immuno-histochemistry, qRT-PCR, and Western blot analysis on xenograft tumors formed by these cell lines (Fig. 3D and E, Supplementary Fig. S2A–B). As expected, L1CAM-suppressed tumor cells showed significantly reduced L1CAM staining compared with control tumor cells. Consistent with our earlier in vitro results, immunohistochemistry revealed that pErk levels were reduced in vivo in L1CAM knockdown animals (Fig. 3F).

L1CAM contributes to metastasis in an orthotopic nude rat model

To determine whether modulating L1CAM expression can influence metastasis in vivo, H460SM-shL1CAM and
control cells were endobronchially implanted into the right caudal lobe airway of nude rats. In both experimental and control groups, all (13 of 13) animals developed regional mediastinal lymph node metastases (Table 2). However, H460SM-shL1CAM animals showed significantly reduced distant metastases to bone (3 of 13) and kidneys (7 of 13), but not to brain and gums, when compared with the control group (P < 0.05; Table 2). No significant differences were noted in the weight of the primary tumors formed by both cell lines. Overall, we observed a significant decrease in the total number of H460SM-shL1CAM animals developing systemic metastasis (8 of 13) compared with the control (P < 0.05; Table 2). These results strongly suggested that L1CAM may potentiate metastasis in lung cancer.

### Discussion

To our knowledge, this is the first study to explore the function of L1CAM in lung cancer both in vitro and in vivo. Here we show that (i) L1CAM expression correlates with clinical outcome in early-stage NSCLC patients; (ii) suppression of L1CAM significantly reduces migration and invasion in three NSCLC cell lines, whereas L1CAM overexpression enhances invasion in noninvasive cells; (iii) L1CAM induces sustained Erk activation under serum stimulation in vitro and in vivo; and (iv) downregulation of L1CAM significantly retards tumor growth in SCID mice and reduces metastasis in nude rats. Collectively, these results are strong evidence that L1CAM plays a pivotal role in lung cancer progression.

Since its discovery in nerve cells 28 years ago, L1CAM expression has emerged as a contributing factor in the progression of a variety of human carcinomas (31). A recent study revealed that elevated L1CAM levels were associated with shorter survival in patients with pancreatic ductal adenocarcinoma (12). In addition, studies on resected tumor samples from colorectal (32), ovarian (9), and melanoma (33) cancer patients have linked L1CAM overexpression to poor prognosis. Our previous work identified L1CAM as a component of a 15-gene signature that predicts prognosis in NSCLC patients (20). Here we confirm the prognostic value of L1CAM expression alone in 4 independent published microarray data sets, thus establishing its robustness as a prognostic marker in NSCLC patients. The result provides a rationale to further explore the role of L1CAM in NSCLC.

Our results show that L1CAM regulates cell migration and invasion in vitro in 3 different histologic subtypes of NSCLC cell lines. Ectopic expression of L1CAM has been reported to modulate haptotactic cell migration on various extracellular matrix (ECM) proteins in melanoma, ovarian, and colon cancer cells (30, 34, 35), and to enhance Matrigel invasion in colon and melanoma cancer cells lacking L1CAM (30, 35). Although L1CAM may play stereotyped roles in mediating neuronal migration in nontransformed cells, diversity in mechanisms underlying tumorigenicity is consistent with L1CAM functioning in a tissue- and context-dependent manner during tumor progression. Li and Galileo (36) showed that L1CAM augmented cell adhesion and migration in breast cancer cell lines, but did not promote Matrigel invasion. In this study, we found that suppression of L1CAM abrogates the ability of NSCLC cells to close an artificial wound and to invade Matrigel.

The mechanisms underlying L1CAM signaling in cancer biology remain controversial. Although L1CAM may induce an epithelial–mesenchymal transition (EMT)-like process in MCF7 breast cancer cells (37), L1CAM-mediated induction of colorectal cancer cell metastasis operates via NF-κB signaling independent of EMT changes (38). Activation of the Erk pathway by L1CAM has been reported previously (39) and is linked to a motile phenotype in fibroblast and melanoma cells (30). Erk signaling is a major determinant in the control of diverse cellular processes such as proliferation, survival, differentiation, and motility and is often upregulated in human cancers (40). Here we show that H125 and H460 cells with L1CAM downregulation are deficient in constitutive Erk1/2 activation upon serum stimulation, which is reversed by reintroduction of shRNA-resistant L1CAM into L1CAM knockdown cells. In addition, xenograft tumors developed from L1CAM knockdown cells show drastically reduced levels of pErk1/2 in vivo. These results highlight the close association of L1CAM activity and the Erk pathway in NSCLC cells.

How L1CAM triggers intracellular Erk signaling to induce a motile and invasive phenotype in lung cancer cells is unknown. One study suggests that direct binding of L1CAM to αvβ5 integrin potentiates haptotactic migration in CHO cells via integrin-mediated signaling events (29).
Cheng and colleagues identified RanBPM as an adapter protein directly linking L1CAM to the Erk pathway in COS-7 cells (41). Therefore, it is conceivable that in response to extracellular stimuli such as serum growth factor, L1CAM interacts with transmembrane binding partners or ECM receptors (e.g., RanBPM, ezrin, and α,β1 integrins), resulting in activation of the Erk pathway and Erk-dependent gene products associated with migratory and invasive phenotypes (38, 41, 42). Silletti and colleagues (30) showed that sustained Erk activation by L1CAM induces Rac1 and cathepsin activity, which directly promote cell migration and invasion of melanoma cells, respectively.

Our data show that L1CAM suppression in H460 and H125 cells reduces tumor growth rate in SCID mice, which can be rescued by restoring L1CAM levels. A role for L1CAM in the tumorigenicity of NSCLC cells is consistent with studies in other cancer cell types, as overexpression of L1CAM in ovarian (34), colon (43), and pancreatic cancer cells (44) augments tumor growth in mice. In addition, biweekly intraperitoneal injection of a monoclonal L1CAM antibody (chCE7) in ovarian carcinoma-bearing mice led to significant reduction of tumor burden (45). Similar effects were recently reported using anti-L1CAM treatment in intrahepatic cholangiocarcinoma-bearing mice (46).

Considering that metastatic spread, not primary tumor burden, is the main cause of lung cancer death, a critical question is whether L1CAM regulates lung cancer metastasis. Our laboratory previously reported that endobronchial implantation of NCI-H460 cells into nude rats generates a primary lung tumor with mediastinal lymph node spread, but rarely systemic metastases (27). Isolation and orthotopic reimplantation of tumor cells from these mediastinal nodes, repeated successively 4 times, produced a variant cell line designated H460SM that spontaneously metastasizes to bone, kidney, brain, soft tissue, and contralateral lung. Orthotopic implantation of lung cancer cells is thought to better mimic the metastatic behavior of lung cancers compared with subcutaneous models (28). Using this model we found that L1CAM suppression significantly reduces metastatic spread of H460SM cells to bone and kidney in nude rats. Proclivity of tumor metastasis for specific organs, such as lung carcinomas for bone, is well documented (47). Our novel finding that L1CAM mediates lung cancer metastasis could account for the strong association between L1CAM expression and poor prognosis of NSCLC patients. Furthermore, our results showing the role of L1CAM in the growth and metastasis of NSCLC supports its potential as a therapeutic target for NSCLC patients in an adjuvant setting.

Considering the role of L1CAM in normal neural development, treatment of antibody-based NSCLC therapeutic strategies at the postnatal stage is unlikely to produce severe neurotoxic effects. L1CAM knockout mice show weak hind limb function due to abnormal corticospinal axon guidance but are viable and have no obvious morphologic defects in major central and peripheral nervous system structures (17, 18). Moreover, tissue-specific alternative exon splicing products functionally relevant isoforms of L1CAM. Neuronal L1CAM differs from L1CAM found in nonneuronal cells in that it contains 4 amino acids (RSLE) in the cytoplasmic domain encoded by the differentially spliced exon 27 (48). Therefore, isoform-specific antibodies directed at the nonneuronal epitope of L1CAM lacking exons 2 and 27 may be an appropriate approach to inhibiting L1CAM protumorigenic function in lung cancer. A recent study showed that combining an L1CAM antibody with chemotherapeutic drugs such as gemcitabine and paclitaxel augmented the response toward chemotherapy alone in preclinical models of pancreatic and ovarian cancer (49). These findings underscore the potential of treatments that use L1CAM antibodies alongside conventional NSCLC adjuvant chemotherapy drugs to improve therapeutic response.

Metastasis is the process that transforms lung cancer from a disease that is local and curable to one that is systemic and fatal. Cell migration and invasion are considered initial steps during metastasis, consistent with our findings that interference with L1CAM expression suppresses both NSCLC metastasis and cell migration and invasion. We further reveal that L1CAM interacts with the Erk signaling pathway to mediate tumor growth, although the details of this mechanism remain to be explored. Supporting these animal studies is our in silico finding that L1CAM expression is consistently an independent prognostic factor in multiple large cohorts of NSCLC patients. Taken together, our data indicate that L1CAM is a key regulator of lung cancer progression and a valuable prognostic and therapeutic target in future lung cancer studies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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