Cancer Letters 447 (2019) 56–65

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

miR-193a-3p inhibition of the Slug activator PAK4 suppresses non-small cell lung cancer aggressiveness via the p53/Slug/L1CAM pathway

Xincheng Liu[a,1], Shengping Min[a,1], Nan Wu[a], Hongli Liu[b], Tao Wang[a], Wei Li[a], Yuanbing Shen[a], Chengling Zhao[a], Hongtao Wang[c], Zhongqing Qian[c], Huanbai Xu[d], Yuqing Chen[a,**], Xiaojing Wang[a,*]

[a] Anhui Clinical and Preclinical Key Laboratory of Respiratory Disease, Department of Respiration, First Affiliated Hospital, Bengbu Medical College, Bengbu, Anhui Province, China
[b] Department of Gynecological Oncology, First Affiliated Hospital, Bengbu Medical College, Bengbu, Anhui Province, China
[c] Department of Immunology, Bengbu Medical College, Bengbu, Anhui Province, China
[d] Department of Endocrinology and Metabolism, Shanghai Jiaotong University Affiliated First People's Hospital, Shanghai, China

ARTICLE INFO

Keywords:
Lung cancer
NSCLC
L1CAM
Slug
PAK4

ABSTRACT

L1 cell adhesion molecule (L1CAM) promotes invasiveness and metastasis in non-small cell lung cancer (NSCLC) cells and is upregulated by the p53-regulated transcription factor Slug. p21-activated kinase 4 (PAK4) directly phosphorylates Slug, resulting in pro-malignant Slug stabilization. We hypothesized that microRNA-based negative regulation of PAK4 would reduce L1CAM-induced NSCLC aggressiveness via destabilizing Slug. We found that elevated L1CAM expression was tightly correlated with p53 loss-of-function and reduced NSCLC patient survival. L1CAM suppression reduced NSCLC cell migration and invasiveness in vitro as well as tumor formation and distal metastasis in vivo. Mechanistically, p53 restricts L1CAM expression through the β-catenin/Slug pathway, with levels of β-catenin and Slug positively correlating with L1CAM expression in NSCLC tumors. The microRNA miR-193a-3p directly targets PAK4 and suppresses downstream p-Slug and L1CAM expression. Silencing PAK4, Slug, and L1CAM mirrored miR-193a-3p's effects upon the migration and invasiveness of NSCLC cells in vitro. Decreased miR-193a-3p levels correlated with elevated PAK4, p-Slug, and L1CAM levels in NSCLC tumors. Our findings support a model of miR-193a-3p as a suppressor of metastatic disease progression in NSCLC via modulation of the p53/Slug/L1CAM pathway.

1. Introduction

Lung cancer is the leading etiology of cancer-linked deaths in the Western world [1–3]. The mortality rates associated with lung cancer have remained stable over the last 30 years, indicating the pressing need for better treatment strategies for this devastating disease [1–3]. Of the different variants of lung cancer, non-small cell lung cancer (NSCLC) is the most common type and accounts for over 80% of all lung cancer cases [1]. The majority of NSCLC cases are diagnosed at later stages following local invasion or distal metastases, when surgical or radiotherapeutic interventions are less effective [4]. Therefore, there is a pressing need for a better understanding of the mechanism(s) underlying NSCLC cell invasiveness and metastasis in order to support novel therapeutic development.

Proteins that regulate cell adhesion and migration are critically involved in promoting NSCLC cell invasiveness and metastasis [4]. One such protein, L1 cell adhesion molecule (L1CAM), is a 220-kilodalton transmembrane protein that plays a key role in cell adhesion and migration [5]. The extracellular domain of L1CAM has numerous immunoglobulin (IgG)-like domains and fibronectin type III repeats, which function in extracellular adhesion, while the cytoplasmic tail mediates intracellular signaling [6]. Notably, L1CAM overexpression has been observed in numerous cancer types and is an independent prognostic indicator in NSCLC patients [7]. On a cellular level, L1CAM

* Corresponding author. Anhui Clinical and Preclinical Key Laboratory of Respiratory Disease, Department of Respiration, First Affiliated Hospital, Bengbu Medical College, No. 287 Changhuai Road, Bengbu, Anhui Province 233000, China.
** Corresponding author. Anhui Clinical and Preclinical Key Laboratory of Respiratory Disease, Department of Respiration, First Affiliated Hospital, Bengbu Medical College, No. 287 Changhuai Road, Bengbu, Anhui Province 233000, China.
E-mail addresses: bbmccyq@126.com (Y. Chen), wangxiaojing8888@163.com (X. Wang).
[1] These authors equally contributed to this work.

https://doi.org/10.1016/j.canlet.2019.01.027
Received 9 October 2018; Received in revised form 12 December 2018; Accepted 21 January 2019
0304-3835/

is involved in the epithelial-mesenchymal transition (EMT) in NSCLC cells, and suppressing L1CAM expression has been shown to inhibit lung NSCLC cell invasiveness in vitro and in vivo [8,9]. Therefore, elucidating the molecular pathway(s) that regulate L1CAM expression in NSCLC cells can improve our understanding of EMT in NSCLC cells and can be beneficial in developing targeted therapies to combat NSCLC aggressiveness.

To that end, Gavert and colleagues discovered that the pro-malignant β-catenin/TCF pathway [10] promotes L1CAM expression [11]. More specifically, the β-catenin/TCF signaling-induced transcription factor Slug [12] has been shown to upregulate L1CAM transcriptional activity [13–16]. Interestingly, the tumor suppressor p53 [17,18] negatively regulates Slug through mouse double minute 2 homolog (MDM2)-mediated degradation of Slug in NSCLC cells [19,20]. Moreover, recent research has revealed that p21-activated kinase 4 (PAK4) directly phosphorylates Slug, resulting in pro-malignant Slug stabilization [21].

These combined findings suggest that microRNA-based negative regulation of PAK4 would reduce L1CAM-induced NSCLC aggressiveness via destabilizing Slug and that targeting Slug via inhibiting PAK4 may be a suitable strategy to combat L1CAM-induced NSCLC aggressiveness. Notably, one microRNA – miR-193a-3p – has been shown to play a tumor-suppressive role in multiple solid tumor types [22–24], including NSCLC [25]. More specifically, miR-193a-3p functions as a tumor suppressor in NSCLC cells via targeting key oncogenes, including KRAS [26,27] and ERBB4 [28,29]. This evidence suggests that miR-193a-3p, and other tumor-suppressive microRNAs, may be valuable targets of investigation for NSCLC researchers.

Here, we hypothesized that miR-193a-3p-based inhibition of PAK4 expression would reduce L1CAM-induced NSCLC aggressiveness via destabilizing Slug. We first confirmed L1CAM's role in NSCLC metastasis, with L1CAM suppression reducing NSCLC cell migration and invasiveness in vitro as well as tumor formation and distal metastasis in vivo. We further demonstrate that elevated L1CAM expression is tightly correlated with p53 loss-of-function and decreased NSCLC patient survival. We also demonstrated that p53 restricts L1CAM expression through the β-catenin/Slug pathway, with levels of β-catenin and Slug positively correlating with L1CAM expression in NSCLC tumors. We discovered miR-193a-3p directly targets PAK4 and suppresses downstream p-Slug and L1CAM expression. We also demonstrated that silencing PAK4, Slug, and L1CAM mirrored miR-193a-3p's effects upon the migration and invasiveness of NSCLC cells in vitro. Moreover, we correlated decreased miR-193a-3p levels with elevated PAK4, p-Slug, and L1CAM levels in NSCLC tumors. Our findings support a model of miR-193a-3p as a suppressor of metastatic NSCLC progression via modulation of the p53/Slug/L1CAM pathway.

2. Materials and methods

The methods used in this study are fully detailed in the Supplementary Information file.

3. Results

3.1. Strong correlation between L1CAM and p53 loss of function and poor prognosis in NSCLC

Primary lung tumor samples and matching normal lung tissue samples were collected from 88 NSCLC patients (Supplementary Table 1) and subjected to quantitative real-time PCR (qPCR) and immunohistochemical analysis. There was a significant increase in L1CAM mRNA expression in NSCLC tumors with p53 loss (n = 28) compared to those with WT p53 (n = 60) (Supp. Fig. 1a). Immunohistochemistry confirmed the association of high L1CAM expression and loss of p53 (Supp. Fig. 1b). Of the 60 cases of p53 WT tumors, only five tumors showed high L1CAM expression (Supp. Fig. 1c). Conversely, the majority of NSCLC cases (23/28) with p53 loss displayed high L1CAM expression. Analysis using Fisher's exact test indicated a significant association between p53 status and L1CAM expression (Supp. Fig. 1c). Survival analysis of NSCLC patients with $L1CAM^{high}$ tumors was significantly lower than the patients with $L1CAM^{low}$ tumors (Supp. Fig. 1d).

3.2. L1CAM stimulates the migration and invasiveness of NSCLC cells in vitro

To assess L1CAM's influence on cell migration and invasiveness, wound healing assays as well as Transwell migration and invasion assays were performed on L1CAM-silenced and L1CAM-overexpressing NSCLC cells. Initially, L1CAM levels were stably modulated in A549 (p53 wild-type), NCI-H1299 (p53 null), and NCI-H1770 (p53 mutant, R248W) cells (Supp. Fig. 2). Wound healing assays indicated that silencing of L1CAM expression significantly suppressed migratory activity, while L1CAM overexpression significantly enhanced migratory activity in all three cell lines (Fig. 1e–g). Collagen IV-coated Transwell assays demonstrated that silencing of L1CAM expression significantly suppressed migration, while L1CAM overexpression significantly enhanced migratory activity in all three cell lines (Fig. 1h). Matrigel-coated Transwell assays showed that silencing of L1CAM expression significantly suppressed invasiveness, while L1CAM overexpression significantly enhanced invasiveness in all three cell lines (Fig. 1i). Notably, no significant effect of L1CAM was observed on NSCLC cell proliferation in vitro (Supp. Fig. 3).

3.3. L1CAM promotes lung tumor initiation, growth, and metastasis in vivo

To assess the effects of L1CAM expression on primary lung tumorigenesis in vivo, a three-vector Sleeping Beauty transposon system (consisting of an inducible, tumorigenic $Kras^{G12C}$ transposon vector, a constitutive shp53 suppression vector, and a constitutive shL1CAM (or ShCtrl) suppression vector) was injected into mice subjects to promote $Kras^{G12C/+}$ shp53 shL1CAM or $Kras^{G12C/+}$ shp53 shCtrl lung tumorigenesis in vivo (Supp. Fig. 4). Six weeks following injection, GFP and dsRed signaling from the lung tissue of infected rats validated successful infection of both $Kras^{G12C}$ and shL1CAM (or shCtrl) vectors, respectively (Fig. 1j). Primary tumorigenesis was quantified in the lung samples. Silencing L1CAM expression significantly suppressed primary tumor initiation in vivo (Fig. 1k).

To assess the effects of L1CAM expression on lung tumor growth and metastasis in vivo, we constructed an endotracheal orthotopic tumor model in nude rats using L1CAM-silenced, L1CAM-overexpressing, and control H460SM cells. Interestingly, altering L1CAM expression had no significant impact on endotracheal orthotopic tumor volumes (Fig. 1l). However, L1CAM knockdown significantly suppressed metastasis to the contralateral (left) lung, bone, gingiva, and kidney (Fig. 1m).

3.4. p53 regulates L1CAM expression in NSCLC cells through an indirect mechanism

To test the effects of exogenous p53 expression on L1CAM expression in p53 null (NCI-H1299) and mutant p53 (NCI-H1770) cells, these cell lines were forced to express functional WT p53, which was confirmed by immunoblotting for p53 and p21 (Fig. 2a). The elevated levels of L1CAM in these cell lines were dramatically reduced following infection with WT p53 compared to control empty vectors (Fig. 2a). To further validate these findings, p53-expressing A549 cancer cells were stably infected with a shRNA to knockdown p53 levels using MLP and pRS retroviral vectors (Fig. 2b). Following knockdown of p53 in WT p53-expressing A549 cells, an increase in L1CAM expression was observed (Fig. 2b). However, neither artificial expression of the two different p53 mutants in p53-null cells nor silencing of mutant p53 expression in p53-mutant cells altered L1CAM expression (Fig. 2c).

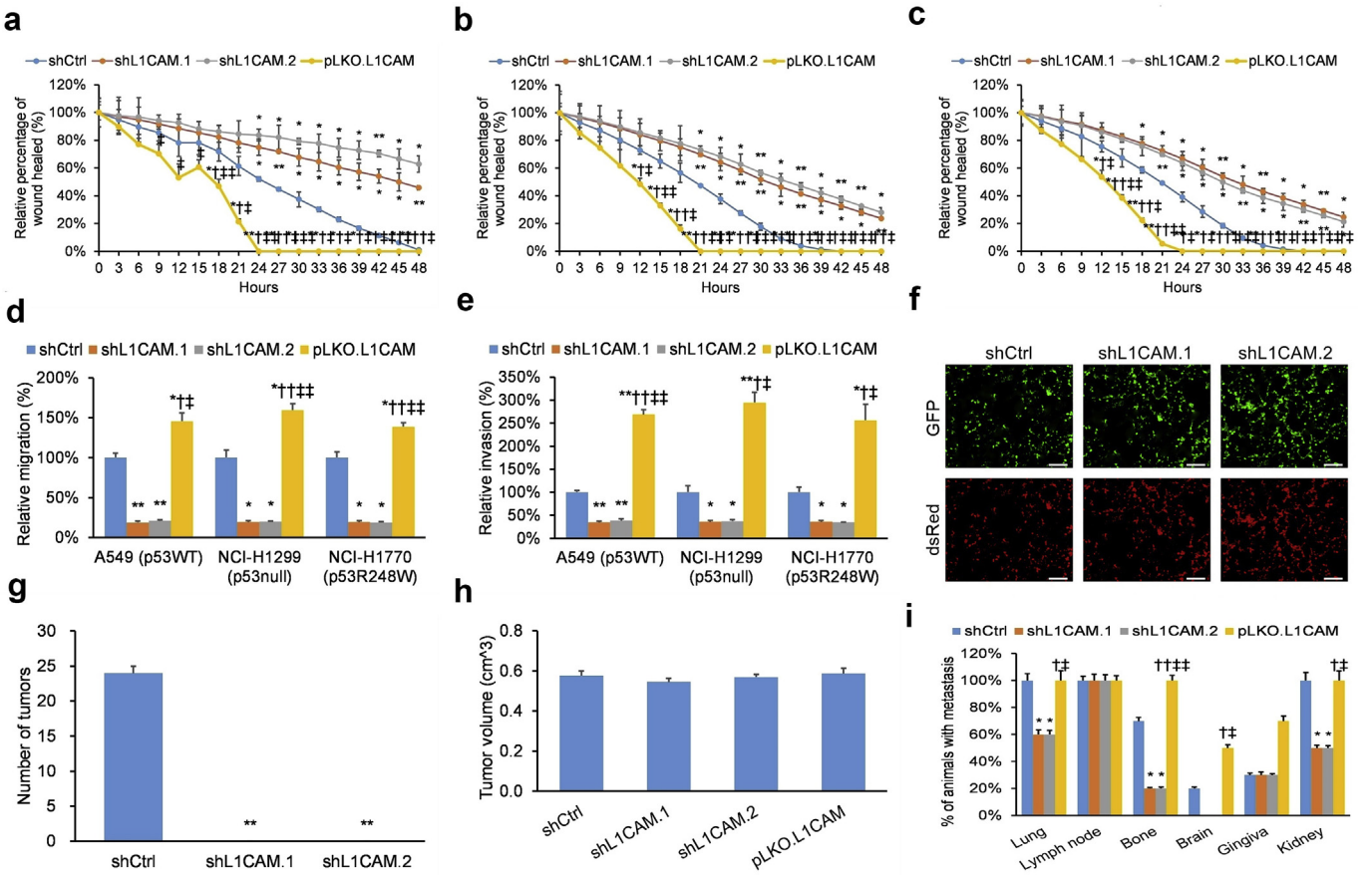


Fig. 1. L1CAM associated with p53 loss-of-function and inferior prognosis in NSCLC; stimulates migration and invasiveness of NSCLC cells in vitro and in vivo. (a–c) Cell front migration assessed by time-lapse wound-healing assays in A549 (p53 wild-type) (a), NCI-H1299 (p53 null) (b), and (c) NCI-H1770 (p53 mutant, R248W) cells. (d) Cell migration assessed by collagen IV-coated Transwell assays in A549 (p53 wild-type), NCI-H1299 (p53 null), and NCI-H1770 (p53 mutant, R248W) cells. (e) Cell invasion assessed by Matrigel-coated Transwell assays in A549 (p53 wild-type), NCI-H1299 (p53 null), and NCI-H1770 (p53 mutant, R248W) cells. (f, g) Results from the murine Sleeping Beauty transposon-based *Kras*$^{G12C/+}$ shp53 lung tumorigenesis model. (f) Representative GFP and dsRed fluorescence images and (g) quantification of tumor counts in lung tissue from transposon-injected mice six weeks following injection (n = 10 mice per group). (h, i) Results from the rat endotracheal orthotopic tumor model. (h) Tumor volumes of primary tumors and (i) quantification of metastatic tumor numbers in nude rats following endobronchial implantation of NSCLC cells (n = 10 rats per group). Data are reported as means ± SEMs. *$p < 0.05$, **$p < 0.01$ vs. shCtrl, †$p < 0.05$, ††$p < 0.01$ vs. shL1CAM.1, ‡$p < 0.05$, ‡‡$p < 0.01$ vs. shL1CAM.2.

Moreover, knocking-down the expression of the two key downstream targets of p53 – p21 and E2F7 – had no effect on the L1CAM-suppressive effects of p53 (Fig. 2d).

3.5. Negative regulation of L1CAM by p53 occurs through a β-catenin/Slug pathway-dependent mechanism

Previous research has shown that WT p53 forms a complex with Slug and MDM2 to promote Slug degradation in NSCLC cells, whereas mutant p53 variants do not promote Slug degradation [19,20]. However, the effect of this p53/Slug pathway on L1CAM expression in NSCLC cells has not been thoroughly investigated.

We ascertained that the promoter region of the L1CAM gene contain Snail/Slug and β-catenin binding sites (Fig. 2e). The presence of Slug and β-catenin enhanced L1CAM promoter 1 activity, while the addition of WT p53 expression suppressed Slug-induced and β-catenin-induced L1CAM promoter 1 activity (Fig. 2f). Moreover, the presence of Slug enhanced L1CAM promoter 2 activity, while the addition of p53WT expression suppressed Slug-induced L1CAM promoter 2 activity (Fig. 2g). Additionally, higher Snail/Slug ratios suppressed L1CAM promoter 1 and 2 activity, while the addition of p53WT expression only suppressed Slug-induced L1CAM promoter 1 and 2 activity at low Snail/Slug ratios (Fig. 2h and i). ChIP analysis demonstrated that the addition of p53WT suppressed Slug binding to several E-box sites on both L1CAM promoters (Fig. 2j). Similarly, the addition of p53WT expression suppressed β-catenin binding to the two binding sites on L1CAM promoter 1 but did not affect binding to the L1CAM promoter 2 binding site (Fig. 2k). Silencing of β-catenin or Slug expression suppressed L1CAM protein expression but did not affect p53 expression (Fig. 2l) and combined silencing of β-catenin and Slug expression suppressed L1CAM protein expression but did not affect p53 expression (Fig. 2m). Co-immunoprecipitation demonstrated that Slug (but not β-catenin) strongly bound to p53 (Fig. 2n). The addition of p53WT suppressed β-catenin and Slug protein expression across all time points (Fig. 2o). To clinically correlate the foregoing in vitro evidence, we found that L1CAM mRNA expression significantly and positively correlated with β-catenin and Slug mRNA levels in human NSCLC tumor samples (Fig. 2p). This combined evidence indicates that p53's negative regulation of L1CAM expression occurs through the β-catenin/Slug pathway.

3.6. miR-193a-3p negatively regulates the Slug/L1CAM pathway via PAK4 suppression

Having demonstrated that the p53/Slug pathway regulates L1CAM expression in NSCLC cells, we aimed at identifying a negative regulator of Slug that may be of clinical relevance in combating L1CAM-induced NSCLC aggressiveness. To that end, microRNAs regulate numerous

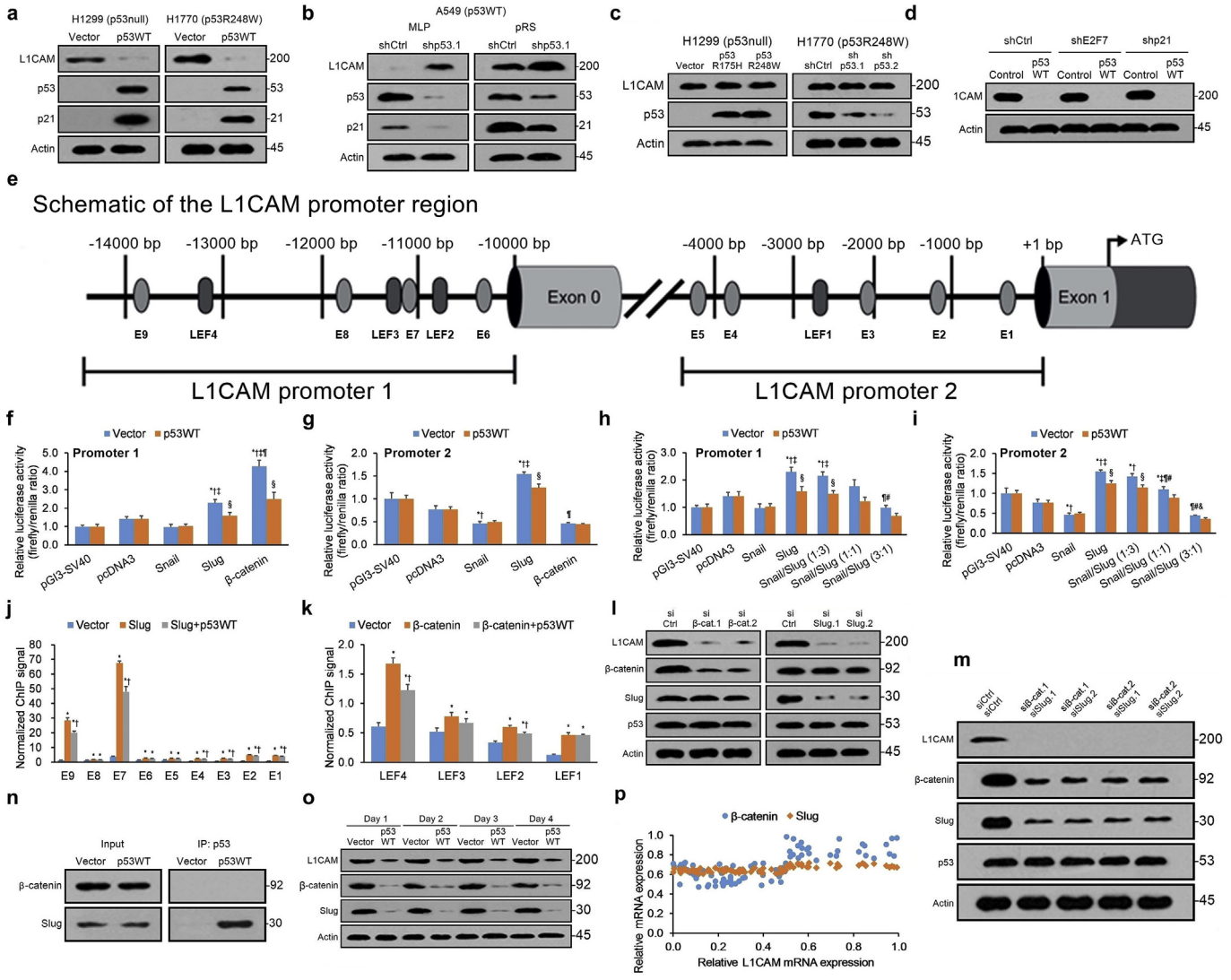


Fig. 2. Regulation of L1CAM expression by p53 in NSCLC cells is β-catenin/Slug-dependent. (a) L1CAM protein expression in NCI-H1299 (p53 null) and NCI-H1770 (p53 mutant, R248W) cells infected with wild-type p53 (p53WT)-expressing vector or a null vector. (b) L1CAM protein expression in A549 (p53WT) cells after shRNA-mediated p53 knockdown via MLP or pRS vectors. (c) L1CAM protein expression in NCI-H1299 (p53 null) cells expressing mutant p53 (R175H, R248W) and NCI-H1770 (p53 mutant, R248W) cells with mutant p53 knockdown. (d) L1CAM protein expression after p53WT re-expression in NCI-H1770 (p53 mutant, R248W) cells with shCtrl, shp21, or shE2F7. (e) Diagram of the two promoter regions of the human L1CAM gene. Snail/Slug-binding sites (termed 'E-boxes') are indicated in light grey, while LEF/TCF/β-catenin binding sites are indicated in dark grey. (f, g) Luciferase assays of the (f) L1CAM promoter 1 construct and (g) L1CAM promoter 2 construct in the presence or absence of wild-type p53 (p53WT) in NCI-H1299 (p53 null) cells. $*p < 0.05$, $**p < 0.01$ vs. pGI3-SV40, $\dagger p < 0.05$, $\dagger\dagger p < 0.01$ vs. pcDNA3, $\ddagger p < 0.05$, $\ddagger\ddagger p < 0.01$ vs. Snail, $\P p < 0.05$, $\P\P p < 0.01$ vs. Slug, $\S p < 0.05$, $\S\S p < 0.01$ vs. matching vector group. (h, i) Luciferase assays of (h) L1CAM promoter 1 construct and (i) L1CAM promoter 2 construct in the presence or absence of wild-type p53 (p53WT) were co-transfected with various Snail/Slug ratios in NCI-H1299 (p53 null) cells. $*p < 0.05$, $**p < 0.01$ vs. pGI3-SV40, $\dagger p < 0.05$, $\dagger\dagger p < 0.01$ vs. pcDNA3, $\ddagger p < 0.05$, $\ddagger\ddagger p < 0.01$ vs. Snail, $\P p < 0.05$, $\P\P p < 0.01$ vs. Slug, $\# p < 0.05$, $\#\# p < 0.01$ vs. Snail/Slug (1:3), $\& p < 0.05$, $\&\& p < 0.01$ vs. Snail/Slug (1:1), $\S p < 0.05$, $\S\S p < 0.01$ vs. matching vector group. (j) ChIP analysis of Slug binding to E-box sites on the two L1CAM promoters in the presence or absence of wild-type p53 (p53WT) in NCI-H1770 (p53 mutant, R248W) cells. $*p < 0.05$, $**p < 0.01$ vs. matching vector group, $\dagger p < 0.05$, $\dagger\dagger p < 0.01$ vs. matching Slug group. (k) ChIP analysis of β-catenin binding to LEF/TCF/β-catenin binding sites on the two L1CAM promoters in the presence or absence of wild-type p53 (p53WT) in NCI-H1770 (p53 mutant, R248W) cells. $*p < 0.05$, $**p < 0.01$ vs. matching vector group, $\dagger p < 0.05$, $\dagger\dagger p < 0.01$ vs. matching β-catenin group. (l) L1CAM protein expression after addition of siRNAs targeting β-catenin or Slug in NCI-H1770 (p53 mutant, R248W) cells. (m) L1CAM protein expression after combined siRNA targeting β-catenin and Slug in NCI-H1770 (p53 mutant, R248W) cells. (n) Co-immunoprecipitation analysis of p53 and β-catenin or Slug after wild-type p53 (p53WT) re-expression or null vector infection in NCI-H1770 (p53 mutant, R248W) cells. (o) L1CAM, β-catenin, and Slug protein expression at various time points after wild-type p53 (p53WT) re-expression or null vector infection in NCI-H1770 (p53 mutant, R248W) cells. (p) Pearson correlation of L1CAM mRNA expression with β-catenin and Slug mRNA expression in NSCLC tumors (L1CAM-β-catenin $r = 0.6898$, L1CAM-Slug $r = 0.6522$). Data are reported as means ± SEMs.

signaling pathways that play key roles in tumorigenesis and metastasis [30]. More specifically, previous bioinformatics analysis on the microRNA miR-193a-3p has revealed that miR-193a-3p negatively regulates several gene targets implicated in promoting invasiveness and metastasis in NSCLC cells [29]. Therefore, we chose to focus on putative miR-193a-3p targets that may influence Slug expression or activation in NSCLC cells.

Through bioinformatics analysis, we first identified a highly-conserved putative miR-193a-3p binding location on the 3′-untranslated region (3′-UTR) of the Slug activator PAK4 [21] (Fig. 3a). Next, we generated firefly luciferase reporter plasmids by inserting the 3′-UTR sequence of PAK4 (PAK4-3′-UTR), which contained the miR-193a-3p

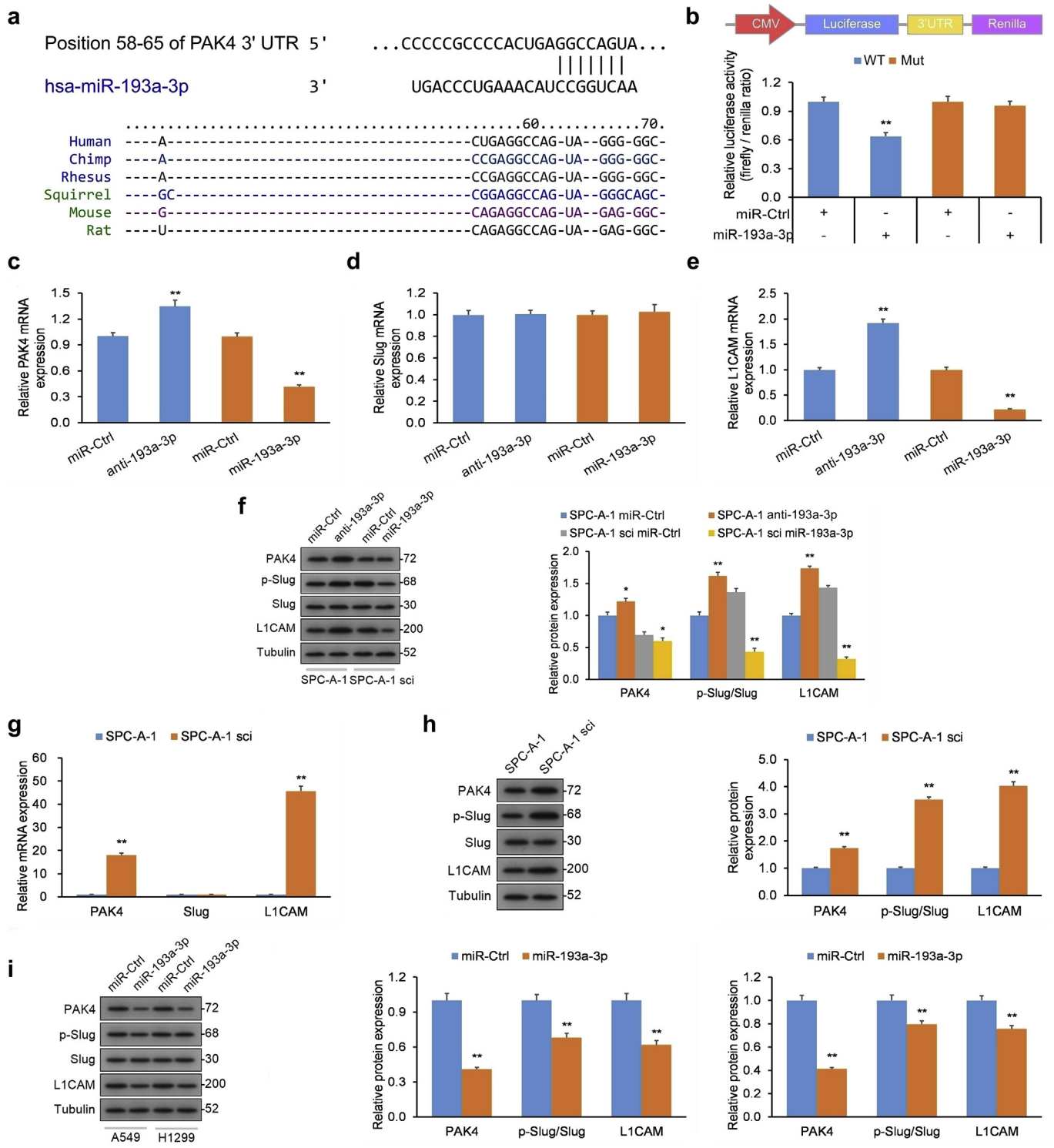


Fig. 3. miR-193a-3p suppresses PAK4/p-Slug/L1CAM signaling via directly targeting the PAK4 3′-UTR. (a) hsa-miR-193a-3p's putative binding location on the human PAK4 3′-UTR (top) and strong conservation of the miR-193a-3p/PAK4 binding sequence among mammalian species (bottom). (b) Relative signal of WT versus mutant PAK4 3′-UTR luciferase reporters in HEK293T cells with co-transfection of miR-193a-3p mimic or scrambled control. The Renilla luciferase signal was used as an internal standardization reference. (c–e) qPCR (mRNA) and (f) WB (protein) analysis of PAK4, p-SLUG, and L1CAM of SPC-A-1sci cells with stable transfection of lentiviral miR-193a-3p mimic or scrambled control, and SPC-A-1 cells with stable transfection of lentiviral miR-193a-3p inhibitor or scrambled control. (g, h) qPCR (mRNA) and WB (protein) analysis of endogenous PAK4, p-SLUG, and L1CAM in SPC-A-1 sci and SPC-A-1 cells. (i) WB analysis of PAK4, p-SLUG, and L1CAM in A549 cells and H1299 cells transiently transfected with miR-193a-3p mimic or scrambled control. Data are reported as means ± SEMs. $*p < 0.05$, $**p < 0.01$ vs. matching Ctrl group.

binding location just downstream of the luciferase gene (Fig. 3b). The corresponding mutant plasmids containing mutant 3′-UTR sequences lacking the putative miR-193a-3p site were also constructed. Co-transfection of the luciferase reporter plasmids into HEK293T cultures was achieved with either miR-193a-3p mimic or scrambled control, and with Renilla vector to serve an internal standardization control.

Overexpression of miR-193a-3p decreased the luminescence signal in HEK293T cells co-transfected with PAK4-3′-UTR, but not in cells co-transfected with mutant 3′-UTR (Fig. 3b).

We then employed a lentiviral infection method to generate SPC-A-1sci cells stably infected with miR-193a-3p mimic (Supp. Fig. 5a) and SPC-A-1 cells stably transfected with a miR-193a-3p inhibitor (anti-193a-3p; Supp. Fig. 5b). We found that overexpression of miR-193a-3p mimic within SPC-A-1sci cells led to a decrease in PAK4 and L1CAM mRNA levels as well as decreases in PAK4, p-Slug, and L1CAM protein levels, while overexpression of anti-193a-3p within SPC-A-1 cells induced the opposite effects (Fig. 3c–f). We also assessed endogenous levels of PAK4, p-Slug, and L1CAM in native SPC-A-1sci and SPC-A-1 cells and found them to be greater in SPC-A-1sci cells, which exhibited higher metastatic potential and lower endogenous miR-193a-3p levels (Fig. 3g and h). Moreover, transient overexpression of miR-193a-3p mimic within A549 cells and H1299 cells induced a decrease in PAK4, p-Slug, and L1CAM protein levels (Fig. 3i). Cumulatively, these experiments demonstrate that miR-193a-3p negatively regulates PAK4, p-Slug, and L1CAM levels in NSCLC cells by directly interacting with the PAK4 3′-UTR.

3.7. miR-193a-3p lowered in NSCLC tumors; correlates with later stage and greater extent of metastasis in NSCLC patients

We measured the levels of miR-193a-3p in 88 patient-derived paired NSCLC tumor and healthy lung specimens, which were assessed in correlation to the diagnosed stage and extent of metastasis in NSCLC patients (Supplementary Table 1). We found that miR-193a-3p was underexpressed in 72% of NSCLC tumor biopsies versus paired healthy lung tissue (Supp. Fig. 6a). We also established that miR-193a-3p expression was approximately halved in NSCLC tumors versus their paired healthy lung tissue (Supp. Fig. 6b). A correlation also emerged between miR-193a-3p levels and the diagnosed stage of NSCLC (Supp. Fig. 6c), with lower miR-193a-3p levels present in later stages of the disease (NSCLC stage III and IV) versus earlier ones (NSCLC stage I and II). An association likewise occurred between miR-193a-3p levels and the extent of metastasis at diagnosis (Supp. Fig. 6d), with miR-193a-3p levels being more downregulated in metastatic disease (NSCLC with lymph node and/or metastasis to distant sites) relative to non-metastatic disease. No significant associations were found between miR-193a-3p levels and NSCLC patient gender and age, or across variations in tumor size, differentiation, and extent of local tumor invasion. Overall, the findings from patient-derived biopsies implicate miR-193a-3p downregulation in the aggressiveness of NSCLC, in terms of both tumor stage and metastasis.

3.8. miR-193a-3p inhibits migration/invasiveness and EMT of NSCLC cells in vitro

Acting on the correlations we observed in NSCLC tumor biopsies, we sought to determine endogenous miR-193a-3p levels and their influence on migration in a panel of human NSCLC cancer cell lines, including A549, SPC-A-1, SPC-A-1sci, LC-21, H358, and H1299. Migration and invasiveness were assessed by Transwell assays (Fig. 4a) and correlated to miR-193a-3p levels (Fig. 4b). We observed that cell lines possessing the lowest levels of endogenous miR-193a-3p also displayed the greatest levels of invasiveness (i.e., A549, SPC-A-1sci, and H1299). Conversely, cell lines with the greatest levels of endogenous miR-193a-3p exhibited the least invasiveness (i.e., SPC-A-1, LC-21, and H358).

We sought to confirm these findings by transiently transfecting cells with a miR-193a-3p mimic or LNA-193a-3p. We performed transfection of SPC-A-1sci with the miR-193a-3p mimic, which resulted in a decrease of migration and invasiveness (Fig. 4c and d). Conversely, the SPC-A-1 cell line, with higher endogenous miR-193a-3p levels, was transfected with LNA-193a-3p, which elicited a rise in migration and invasiveness (Fig. 4c, e). We also performed miR-193a-3p mimic transfection of the cell lines that exhibited the lowest endogenous miR-193a-3p levels (i.e., A549 and H1299), which resulted in a decrease of migration and invasiveness (Supp. Fig. 7).

Stable expression of the miR-193a-3p mimic attenuated the invasiveness of SPC-A-1sci cells as determined by a scratch assay (Fig. 4f and g). On the other hand, stable expression of anti-193a-3p within SPC-A-1 cultures boosted invasiveness of SPC-A-1 cells (Fig. 4f, h). Moreover, stable infection of the miR-193a-3p mimic into SPC-A-1sci cells induced an alteration from the aggressive spindle-like or star-like phenotype to the more stable round cobblestone-shaped phenotype (Supp. Fig. 8a). On the other hand, stable infection of anti-193a-3p into SPC-A-1 cells elicited the opposite effect (Supp. Fig. 8a). These scratch assays and morphological analyses imply that miR-193a-3p suppresses EMT progression in NSCLC cells.

To corroborate these findings, we conducted an analysis of E-cadherin (an epithelial characteristic marker) and vimentin (a mesenchymal characteristic marker) expression by qPCR and Western blot (WB) assays. Stable infection of the miR-193a-3p mimic into SPC-A-1sci cells boosted E-cadherin protein (Fig. 4i and j) and mRNA (Supp. Fig. 8b) levels, while it reduced vimentin protein (Fig. 4i and j) and mRNA (Supp. Fig. 8c) levels, implying miR-193a-3p's inhibition of EMT in NSCLC cells. Conversely, stable infection of anti-193a-3p into SPC-A-1 cells reduced E-cadherin protein (Fig. 4i, k) and mRNA (Supp. Fig. 8d) levels, while it boosted vimentin protein (Fig. 4i, k) and mRNA (Supp. Fig. 8e) levels. Overall, this evidence demonstrates that miR-193a-3p impedes migration/invasiveness and EMT in NSCLC cells.

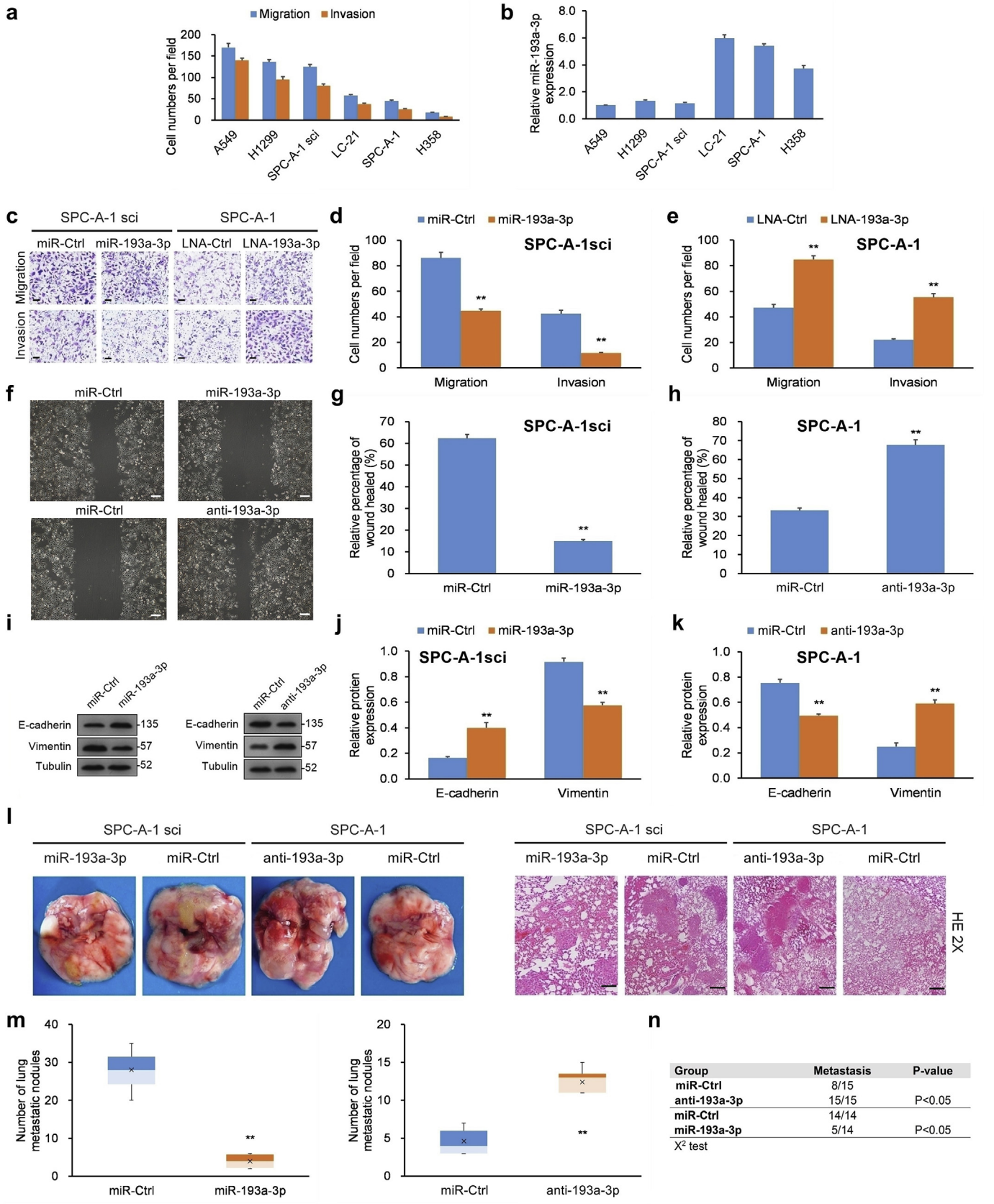
3.9. miR-193a-3p attenuates metastasis of murine xenograft NSCLC tumors

EMT is believed to initiate metastasis during in vivo cancer progression [31]. Therefore, following our in vitro findings that miR-193a-3p inhibits EMT, we sought to determine whether it impacts metastasis of NSCLC cells in vivo. We performed lateral tail vein injection of stably-transfected SPC-A-1sci cells (with miR-193a-3p-mimic or scrambled control) or stably-transfected SPC-A-1 cells (with anti-193a-3p or scrambled control) in nude mice (n = 12 per cohort). As anticipated, there was a lower count of metastatic tumor nodules in the lungs of miR-193a-3p-mimic mice and a higher count in the lungs of anti-193a-3p mice versus their respective controls (Fig. 4l and m). The frequency of lung metastases was lesser in the miR-193a-3p-mimic cohort and greater in the anti-193a-3p cohort versus their respective controls (Fig. 4n). In accordance with the in vitro findings, these studies in mice implicate miR-193a-3p as an inhibitor of metastasis in NSCLC.

3.10. miR-193a-3p mediates its influence on migration/invasiveness of NSCLC cells through PAK4, p-Slug, and L1CAM

To determine their role in migration and invasion, we transiently knocked-down (KD) expression of endogenous PAK4, Slug, and L1CAM in SPC-A-1sci cells by utilizing small interfering RNAs (siRNAs). All siRNAs were effective and induced a marked decrease in mRNA and protein levels for PAK4, Slug, and L1CAM in SPC-A-1sci cells (Supp. Fig. 9).

KD of PAK4, Slug, or L1CAM induced a decrease in migration and invasion as assessed by Transwell assays (Fig. 5a and b). To determine whether the impact of PAK4, Slug, and L1CAM KD on migration and invasion was mediated via miR-193a-3p, we devised a co-transfection experiment within SPC-A-1 cells of siRNAs and LNA-193a-3p (Fig. 5c–f). In all instances, we saw robust decrease in PAK4, p-Slug, and L1CAM protein levels (Fig. 5c–f). The promotion of migration and invasion by LNA-193a-3p was partly reversed in siPAK4, siSlug, and siL1CAM SPC-A-1sci cultures (Fig. 5g and h). This dependence implies that PAK4, Slug, and L1CAM mediate, to some extent, the impact of miR-193a-3p on the invasiveness of NSCLC cells.



(caption on next page)

Fig. 4. miR-193a-3p inhibits migration/invasiveness and EMT of NSCLC cells in vitro and in vivo. (a) Migration and invasion were measured by a Transwell assay for the panel of human NCSLC cancer cell lines (A549, SPC-A-1sci, SPC-A-1, LC-21, H1299, and H358). (b) Endogenous miR-193a-3p transcript levels in the NSCLC panel were assessed by qPCR and normalized to U6 RNA transcripts. (c) Migration and invasion were measured by a Transwell assay (scale bars = 100 μm) for (d) SPC-A-1sci cells transiently transfected with miR-193a-3p mimic or scrambled control, and (e) SPC-A-1 cells transiently transfected with miR-193a-3p LNA inhibitor or negative control LNA. (f) Scratch assay (scale bars = 100 μm) for (g) SPC-A-1sci cells with stable transfection of lentiviral miR-193a-3p mimic or scrambled control and (h) SPC-A-1 cells with stable transfection of lentiviral miR-193a-3p inhibitor or scrambled control. (i) Western blot (WB) of E-cadherin and vimentin protein levels normalized to β-actin for (j) SPC-A-1sci cells with stable transfection of lentiviral miR-193a-3p mimic or scrambled control and (k) SPC-A-1 cells with stable transfection of lentiviral miR-193a-3p inhibitor or scrambled control. (l) Typical images of murine lung tumors and histological sections (scale bars = 200 μm) therefrom seven weeks after injection of SPC-A-1sci cells transiently transfected with miR-193a-3p mimic or scrambled control, and SPC-A-1 cells transiently transfected with miR-193a-3p LNA inhibitor or negative control LNA. (m) Quantitative analysis of microscopic metastatic tumor nodules in the lungs of mice (n = 12 per cohort). (n) Frequency of metastasis post tail vein injection in the lungs of mice (n = 12 per cohort) is tabulated. Data are reported as means ± SEMs. $*p < 0.05$, $**p < 0.01$ vs. matching Ctrl group.

3.11. miR-193a-3p inversely associated with PAK4/p-Slug/L1CAM pathway expression in NSCLC

Earlier, we established a correlation between lower miR-193a-3p levels with more advanced and metastatic NSCLC in patient-derived tumor biopsies. Since we correlated increased miR-193a-3p levels with lowered PAK4, p-Slug, and L1CAM levels in vitro, we sought to validate this relationship in patient-derived NSCLC tumor biopsy samples. As anticipated, PAK4, p-Slug, and L1CAM protein levels were higher in tumor biopsy versus matching healthy lung samples (Fig. 5i–k). Furthermore, PAK4, p-Slug, and L1CAM protein levels were greater in tumor biopsies from NSCLC patients with metastatic disease (either to

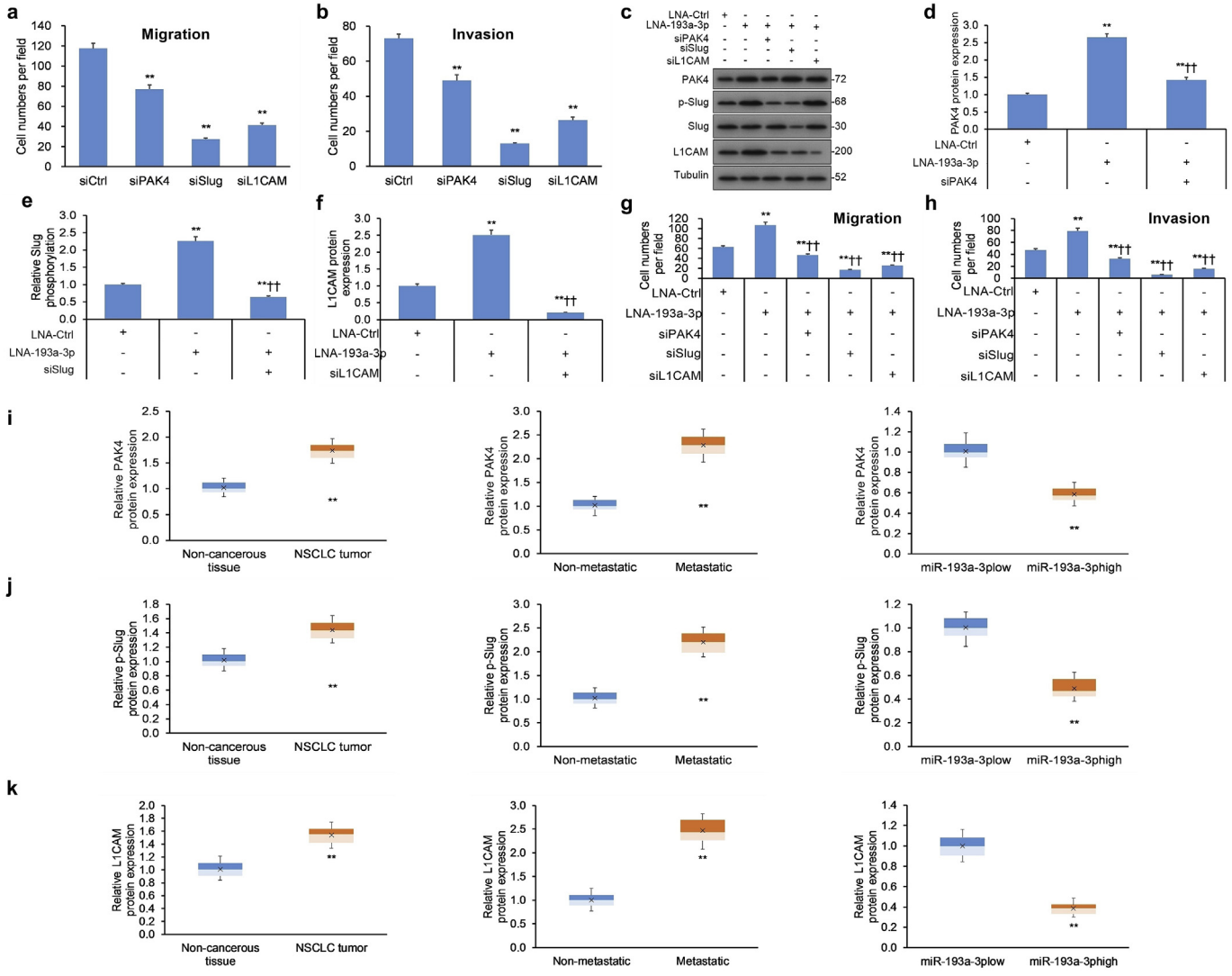


Fig. 5. miR-193a-3p mediates its influence on migration and invasion through PAK4/p-Slug/L1CAM signaling. (a) Migration and (b) invasion were measured by a Transwell assay in siPAK4, siSlug, and siL1CAM SPC-A-1sci cells versus negative controls. (c) WB analysis, normalized to β-actin, of (d) PAK4, (e) p-Slug, and (f) L1CAM levels in siPAK4, siSlug, and siL1CAM SPC-A-1 cultures with miR-193a-3p mimic or inhibitor versus scrambled controls. (g) Migration and (h) invasion were measured by a Transwell assay in siPAK4, siSlug, and siL1CAM SPC-A-1 cultures with miR-193a-3p mimic or inhibitor versus scrambled controls. $*p < 0.05$, $**p < 0.01$ vs. matching Ctrl group. $†p < 0.05$, $††p < 0.01$ vs. matching LNA-193a-3p group. (i–k) WB (protein) analysis of endogenous (i) PAK4, (j) p-Slug, and (k) L1CAM levels in patient-derived biopsies stratified by tumor status, metastasis status, and miR-193a-3p levels. $*p < 0.05$, $**p < 0.01$. Data are reported as means ± SEMs.

lymph nodes or distal sites) than from NSCLC patients with non-metastatic disease (Fig. 5i–k). Moreover, increased PAK4, p-Slug, and L1CAM protein levels were linked to decreased miR-193a-3p levels (Fig. 5i–k).

4. Discussion

Metastasis is one of the key hallmarks of aggressive malignancies and is the leading cause of NSCLC-related mortality, with roughly 90% of NSCLC patients succumbing to metastasis as opposed to their primary tumors [32,33]. Thus, cancer researchers need to better elucidate the molecular mechanism(s) underlying NSCLC metastasis. Previous work has shed light on L1CAM's role in NSCLC metastasis, with L1CAM suppression reducing NSCLC cell motility and invasiveness in vitro as well as tumor formation and distal metastasis in vivo [34]. Our current findings corroborate these previous findings and further demonstrate that elevated L1CAM expression is tightly correlated with p53 loss-of-function and reduced NSCLC patient survival. We also found that shRNA-mediated L1CAM suppression significantly reduced tumor initiation in a murine $Kras^{G12C/+}$ shp53-driven lung tumorigenesis model. Interestingly, a recently published orthotopic shRNA screening study performed on $Kras^{G12D/+}$;$Trp53^{-/-}$ cells revealed that L1CAM expression is required for NSCLC cell proliferation under inflammatory conditions, and L1CAM overexpression increases the metastatic potential of $Kras^{G12D/+}$;$Trp53^{-/-}$ tumors in vivo [35]. These previous findings indicate that L1CAM is a key oncogene driving $Kras^{G12D/+}$;$Trp53^{-/-}$ tumor progression and concord with the results from our murine $Kras^{G12C/+}$ shp53-driven lung tumorigenesis model.

We further demonstrated that p53 restricts L1CAM expression through the β-catenin/Slug pathway, with levels of β-catenin and Slug positively correlating with L1CAM expression in NSCLC tumors. Our findings concord with other studies showing that p53 promotes Slug degradation in NSCLC cells through the p53/MDM2 complex [19,20]. These initial findings revealed that the p53/Slug pathway is responsible for regulating L1CAM expression in NSCLC cells, so we aimed at identifying a negative regulator of Slug that may be of clinical relevance in combating L1CAM-induced NSCLC aggressiveness. To that end, we focused on the mature miRNA miR-193a, which has already been shown to be negatively correlated with NSCLC tumor metastasis and to inhibit the migration/invasiveness of NSCLC cells in vitro and metastasis in vivo [29]. We also found that miR-193a-3p significantly diminished NSCLC cell migration/invasiveness and EMT in vitro and reduced tumor metastasis in vivo; moreover, we associated lower miR-193a-3p levels with NSCLC tumor aggressiveness, in terms of both tumor stage and metastasis.

That being said, miR-193a-3p's target genes and their roles in NSCLC metastasis remain largely unknown. Applying bioinformatics analysis and in vitro experiments, we identified miR-193a-3p as a direct negative regulator of the Slug activator PAK4 [21], whereby miR-193a-3p suppresses downstream p-Slug and L1CAM expression. Importantly, we also demonstrated that silencing PAK4, Slug, and L1CAM mirrored miR-193a-3p's effects upon the migration and invasiveness of NSCLC cells in vitro. Moreover, we correlated decreased miR-193a-3p levels with elevated PAK4, p-Slug, and L1CAM levels in NSCLC tumors. PAK4, as a key effector of the Rho family of GTPases, is upregulated in several *Ras*-driven cancers [36] and regulates several cellular functions related to tumorigenesis and metastasis [37]. Consistent with our current findings showing miR-193a-3p's suppression of EMT marker expression, PAK4 inhibition by PAK4 allosteric modulators (PAMs) has been shown to suppress EMT marker expression (EpCAM, vimentin, and Snail) and xenograft tumor growth [38]. A thorough PAK4 interactome analysis has revealed that PAK4 directly interacts with 313 proteins primarily associated with the actin cytoskeleton, the replication fork, 14-3-3, and the proteasome [39]; most notably, PAK4's regulation of actin cytoskeleton proteins has been shown to affect cell morphology, adhesion, and migration [37]. Therefore, the observed effects of miR-193a-3p's repression of PAK4 expression may be partly due to limiting PAK4's effects on pro-malignant signaling pathways other than p53/Slug/L1CAM.

These combined findings support our proposed model of miR-193a-3p as a suppressor of metastatic disease progression in NSCLC via modulation of the p53/Slug/L1CAM pathway (Supp. Fig. 10). In normal cells, WT p53 promotes ubiquitin/proteasome-mediated Slug degradation [19], thereby suppressing L1CAM expression as well as downstream invasiveness and metastatic activity. However, in p53-null NSCLC cells, p53 loss-of-function enables Slug stabilization while miR-193a-3p underexpression promotes PAK4-mediated Slug activation, thereby promoting L1CAM expression as well as downstream invasiveness and metastatic activity. Moreover, as there is evidence suggesting that PAK4 phosphorylation of Slug protects Slug from ubiquitin/proteasome-mediated degradation [21], PAK4 upregulation from miR-193a-3p underexpression may also promote Slug stabilization in $p53^{+/+}$ NSCLC cells.

In conclusion, our results support miR-193a-3p's role in decreasing the metastatic potential of NSCLC cells via regulation of p53/Slug/L1CAM signaling. As miR-193a-3p appears to function as an anti-metastatic miRNA in NSCLC, miR-193a-3p agomiR therapies (and other PAK4-targeting therapies) could show promise in suppressing the invasiveness and metastasis of NSCLC tumor cells.

Ethics approval and consent to participate

All human and animal studies were reviewed and approved by the appropriate committees at Bengbu Medical Hospital (see methods). All patients provided informed consent for use of their samples in this study.

Consent for publication

No personal patient data were used in this manuscript, and no identifiers linking data to specific patients were provided. All authors have read and approved the publication of this manuscript.

Competing interests

The authors declare no competing interests associated with the publication of this manuscript.

Data availability

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (grant no. 81772493), the Key Program of Natural Science Research of Higher Education of Anhui Province (grant no. KJ2017A241) and the Science and Technology Program of Anhui Province (grant nos. YDZX20183400002554).

List of abbreviations

NSCLC	Non-small cell lung cancer
qPCR	quantitative polymerase chain reaction
EMT	epithelial-mesenchymal transition
Erk	extracellular signal-regulated kinase
GADD45	growth arrest and DNA damage-inducible 45
BAI1	brain-specific angiogenesis inhibitor 1
MDM2	mouse double minute 2 homolog
H&E	hematoxylin and eosin
IHC	Immunohistochemistry

ATCC	American Type Culture Collection
RPMI	Roswell Park Memorial Institute
FBS	fetal bovine serum
SDS	sodium dodecyl sulfate

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.01.027.

References

[1] L.A. Torre, R.L. Siegel, A. Jemal, Lung cancer statistics, Adv. Exp. Med. Biol. 893 (2016) 1–19.
[2] P.G. Thill, P. Goswami, G. Berchem, B. Domon, Lung cancer statistics in Luxembourg from 1981 to 2008, Bull. Soc. Sci. Med. Grand-Duche Luxemb. (2011) 43–55.
[3] M. Zwitter, Dutch statistics on lung cancer: sobering experience for a new approach, J. Thorac. Oncol.: Offic. Publ. Int. Assoc. Study Lung Canc. 7 (2012) 269–271.
[4] F. Perlikos, K.J. Harrington, K.N. Syrigos, Key molecular mechanisms in lung cancer invasion and metastasis: a comprehensive review, Crit. Rev. Oncol.-Hematol. 87 (2013) 1–11.
[5] J. Lindner, F.G. Rathjen, M. Schachner, L1 mono- and polyclonal antibodies modify cell migration in early postnatal mouse cerebellum, Nature 305 (1983) 427–430.
[6] M.K. Schafer, P. Altevogt, L1CAM malfunction in the nervous system and human carcinomas, Cell. Mol. Life Sci. 67 (2010) 2425–2437.
[7] V. Tischler, M. Pfeifer, S. Hausladen, U. Schirmer, A.K. Bonde, G. Kristiansen, M.L. Sos, W. Weder, H. Moch, P. Altevogt, A. Soltermann, L1CAM protein expression is associated with poor prognosis in non-small cell lung cancer, Mol. Canc. 10 (2011) 127.
[8] K. Doberstein, P.N. Harter, U. Haberkorn, N.P. Bretz, B. Arnold, R. Carretero, G. Moldenhauer, M. Mittelbronn, P. Altevogt, Antibody therapy to human L1CAM in a transgenic mouse model blocks local tumor growth but induces EMT, Int. J. Canc. 136 (2015) E326–E339.
[9] H. Kiefel, S. Bondong, M. Pfeifer, U. Schirmer, N. Erbe-Hoffmann, H. Schafer, S. Sebens, P. Altevogt, EMT-associated up-regulation of L1CAM provides insights into L1CAM-mediated integrin signalling and NF-kappaB activation, Carcinogenesis 33 (2012) 1919–1929.
[10] R. Nusse, H. Clevers, Wnt/β-catenin signaling, disease, and emerging therapeutic modalities, Cell 169 (2017) 985–999.
[11] N. Gavert, M. Conacci-Sorrell, D. Gast, A. Schneider, P. Altevogt, T. Brabletz, A. Ben-Ze'ev, L1, a novel target of β-catenin signaling, transforms cells and is expressed at the invasive front of colon cancers, J. Cell Biol. 168 (2005) 633–642.
[12] A. Ben-Ze'ev, M. Shtutman, J. Zhurinsky, The integration of cell adhesion with gene expression: the role of β-catenin, Exp. Cell Res. 261 (2000) 75–82.
[13] C. Geismann, M. Morscheck, D. Koch, F. Bergmann, H. Ungefroren, A. Arlt, M.-S. Tsao, M.G. Bachem, P. Altevogt, B. Sipos, Up-regulation of L1CAM in pancreatic duct cells is transforming growth factor β1–and slug-dependent: role in malignant transformation of pancreatic cancer, Cancer Res. 69 (2009) 4517–4526.
[14] M. Pfeifer, U. Schirmer, C. Geismann, H. Schäfer, S. Sebens, P. Altevogt, L1CAM expression in endometrial carcinomas is regulated by usage of two different promoter regions, BMC Mol. Biol. 11 (2010) 64.
[15] C. Geismann, A. Arlt, I. Bauer, M. Pfeifer, U. Schirmer, P. Altevogt, S.S. Müerköster, H. Schäfer, Binding of the transcription factor Slug to the L1CAM promoter is essential for transforming growth factor-β1 (TGF-β)-induced L1CAM expression in human pancreatic ductal adenocarcinoma cells, Int. J. Oncol. 38 (2011) 257–266.
[16] K. Lund, J.L. Dembinski, N. Solberg, A. Urbanucci, I.G. Mills, S. Krauss, Slug-dependent upregulation of L1CAM is responsible for the increased invasion potential of pancreatic cancer cells following long-term 5-FU treatment, PLoS One 10 (2015) e0123684.
[17] A.J. Levine, p53, the cellular gatekeeper for growth and division, Cell 88 (1997) 323–331.
[18] T. Riley, E. Sontag, P. Chen, A. Levine, Transcriptional control of human p53-regulated genes, Nat. Rev. Mol. Cell Biol. 9 (2008) 402–412.
[19] S.-P. Wang, W.-L. Wang, Y.-L. Chang, C.-T. Wu, Y.-C. Chao, S.-H. Kao, A. Yuan, C.-W. Lin, S.-C. Yang, W.-K. Chan, p53 controls cancer cell invasion by inducing the MDM2-mediated degradation of Slug, Nat. Cell Biol. 11 (2009) 694.
[20] D. Wu, M. Lee, J. Wang, C. Chen, Y. Cheng, H. Lee, DDX3 loss by p53 inactivation promotes tumor malignancy via the MDM2/Slug/E-cadherin pathway and poor patient outcome in non-small-cell lung cancer, Oncogene 33 (2014) 1515.
[21] J.-J. Park, M.-H. Park, E.H. Oh, N.-K. Soung, S.J. Lee, J.-K. Jung, O.-J. Lee, S.J. Yun, W.-J. Kim, E.-Y. Shin, The p21-activated kinase 4-Slug transcription factor axis promotes epithelial− mesenchymal transition and worsens prognosis in prostate cancer, Oncogene (2018) 1.
[22] M. Williams, M.B. Kirschner, Y.Y. Cheng, J. Hanh, J. Weiss, N. Mugridge, C.M. Wright, A. Linton, S.C. Kao, J.J.B. Edelman, miR-193a-3p is a potential tumor suppressor in malignant pleural mesothelioma, Oncotarget 6 (2015) 23480.
[23] J.-E. Kwon, B.-Y. Kim, S.-Y. Kwak, I.-H. Bae, Y.-H. Han, Ionizing radiation-inducible microRNA miR-193a-3p induces apoptosis by directly targeting Mcl-1, Apoptosis 18 (2013) 896–909.
[24] K.-i. Kozaki, I. Imoto, S. Mogi, K. Omura, J. Inazawa, Exploration of tumor-suppressive microRNAs silenced by DNA hypermethylation in oral cancer, Cancer Res. 68 (2008) 2094–2105.
[25] W. Deng, M. Yan, T. Yu, H. Ge, H. Lin, J. Li, Y. Liu, Q. Geng, M. Zhu, L. Liu, Quantitative proteomic analysis of the metastasis-inhibitory mechanism of miR-193a-3p in non-small cell lung cancer, Cell. Physiol. Biochem. 35 (2015) 1677–1688.
[26] E.G. Seviour, V. Sehgal, D. Mishra, R. Rupaimoole, C. Rodriguez-Aguayo, G. Lopez-Berestein, J.-S. Lee, A.K. Sood, M.P. Kim, G.B. Mills, Targeting KRas-dependent tumour growth, circulating tumour cells and metastasis in vivo by clinically significant miR-193a-3p, Oncogene 36 (2017) 1339.
[27] Q. Fan, X. Hu, H. Zhang, S. Wang, H. Zhang, C. You, C.-Y. Zhang, H. Liang, X. Chen, Y. Ba, MiR-193a-3p is an important tumour suppressor in lung cancer and directly targets KRAS, Cell. Physiol. Biochem. 44 (2017) 1311–1324.
[28] H. Liang, M. Liu, X. Yan, Y. Zhou, W. Wang, X. Wang, Z. Fu, N. Wang, S. Zhang, Y. Wang, miR-193a-3p functions as a tumor suppressor in lung cancer by down-regulating ERBB4, J. Biol. Chem. 290 (2015) 926–940.
[29] T. Yu, J. Li, M. Yan, L. Liu, H. Lin, F. Zhao, L. Sun, Y. Zhang, Y. Cui, F. Zhang, MicroRNA-193a-3p and-5p suppress the metastasis of human non-small-cell lung cancer by downregulating the ERBB4/PIK3R3/mTOR/S6K2 signaling pathway, Oncogene 34 (2015) 413.
[30] R. Rupaimoole, F.J. Slack, MicroRNA therapeutics: towards a new era for the management of cancer and other diseases, Nat. Rev. Drug Discov. 16 (2017) 203.
[31] T. Brabletz, R. Kalluri, M.A. Nieto, R.A. Weinberg, EMT in cancer, Nat. Rev. Canc. 18 (2018) 128.
[32] W. Sharieff, G. Okawara, T. Tsakiridis, J. Wright, Predicting 2-year survival for radiation regimens in advanced non-small cell lung cancer, Clin. Oncol. 25 (2013) 697–705.
[33] R.S. Heist, J.A. Engelman, SnapShot: non-small cell lung cancer, Cancer Cell 21 (2012) 448 e442.
[34] J. Hai, C.Q. Zhu, B. Bandarchi, Y.H. Wang, R. Navab, F.A. Shepherd, I. Jurisica, M.S. Tsao, L1 cell adhesion molecule promotes tumorigenicity and metastatic potential in non-small cell lung cancer, Clin. Canc. Res. 18 (2012) 1914–1924.
[35] M. Serresi, B. Siteur, D. Hulsman, M.J. Schmitt, C. Lieftink, B. Morris, M. Cesaroni, N. Proost, R.L. Beijersbergen, M. van Lohuizen, Ezh2 inhibition in Kras-driven lung cancer amplifies inflammation and associated vulnerabilities, J. Exp. Med. 215 (2018) 3115–3135.
[36] M. Radu, G. Semenova, R. Kosoff, J. Chernoff, PAK signalling during the development and progression of cancer, Nat. Rev. Canc. 14 (2014) 13.
[37] B.H. Ha, E.M. Morse, B.E. Turk, T.J. Boggon, Signaling, regulation and specificity of the type II p21-activated kinases, J. Biol. Chem. 290 (2015) 12975–12983.
[38] A.S. Azmi, I. Muqbil, A. Aboukameel, W. Senapedis, E. Baloglu, Y. Landesman, S. Shacham, M. Kauffman, P.A. Philip, R.M. Mohammad, The Role of P21-Activated Kinase 4 (PAK4) in Cancer Stemness and Epithelial-To-Mesenchymal Transition, AACR, 2016.
[39] M. Zhao, M. Spiess, H.J. Johansson, H. Olofsson, J. Hu, J. Lehtiö, S. Strömblad, Identification of the PAK4 interactome reveals PAK4 phosphorylation of N-WASP and promotion of Arp2/3-dependent actin polymerization, Oncotarget 8 (2017) 77061.