The CRTC1-NEDD9 Signaling Axis Mediates Lung Cancer Progression Caused by LKB1 Loss

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Abstract

Somatic mutation of the tumor suppressor gene LKB1 occurs frequently in lung cancer where it causes tumor progression and metastasis, but the underlying mechanisms remain mainly unknown. Here, we show that the oncogene NEDD9 is an important downstream mediator of lung cancer progression evoked by LKB1 loss. In de novo mouse models, RNAi-mediated silencing of Nedd9 inhibited lung tumor progression, whereas ectopic NEDD9 expression accelerated this process. Mechanistically, LKB1 negatively regulated NEDD9 transcription by promoting cytosolic translocation of CRTC1 from the nucleus. Notably, ectopic expression of either NEDD9 or CRTC1 partially reversed the inhibitory function of LKB1 on metastasis of lung cancer cells. In clinical specimens, elevated expression of NEDD9 was associated with malignant progression and metastasis. Collectively, our results decipher the mechanism through which LKB1 deficiency promotes lung cancer progression and metastasis, and provide a mechanistic rationale for therapeutic attack of these processes. Cancer Res; 72(24); 6502-11. ©2012 AACR.

Introduction

Lung cancer is one of the most deadly diseases worldwide, mainly attributing to the high frequency of metastasis (1). Somatic inactivating mutation of LKB1 is frequently observed in approximately 30% of non–small cell lung cancer (NSCLC; refs. 2–8) as well as several other epithelial carcinomas including prostate cancer, cervix cancer, and pancreas cancer (9). Our previous work in mouse models has uncovered an essential role of LKB1 loss in promoting lung cancer progression and metastasis (4). However, the molecules mediating lung cancer progression and metastasis triggered by LKB1 loss remain elusive. Identifying such mediators and deciphering the underlying mechanism are important for better understanding the cancer progression and metastasis process, as well as finding potential therapeutic targets for effective lung cancer treatment in clinic.

As a master serine/threonine kinase and tumor suppressor, LKB1 phosphorylates more than a dozen of downstream kinases including the well-known energy gauge kinase adenosine monophosphate kinase involved in mTOR signaling pathway (10). We have recently shown that the activation of mTOR signaling pathway by LKB1 loss turns on lysyl oxidase (LOX) gene expression, which promotes lung cancer progression and metastasis through extracellular matrix remodeling (11). However, only partial inhibition of tumor progression was achieved by either LOX enzymatic inhibitor treatment or the combinational therapy using phosphatidylinositol 3-kinase-mTOR and mitogen-activated protein–extracellular signal-regulated kinase inhibitors (11, 12). Considering the limited efficacy of targeting either LOX or mTOR signaling pathway, we reason there may exist other pathways or molecules playing essential roles in lung cancer progression and metastasis induced by LKB1 loss.

Through microarray data analysis, we have previously identified several potential candidates associated with metastasis in Lkb1-deficient murine lung cancer, including Vegf and Nedd9 (neural precursor cell expressed, developmentally downregulated 9, also known as HEF1, Cas-L; ref. 4). While we have previously shown in mice that blocking Vegf signaling is not effective in inhibition of Lkb1-deficient lung tumor metastasis (13), additional data support a potential role for Nedd9. NEDD9 is a noncatalytic scaffold protein (14–16), and has been implicated in the metastatic behavior of several types of solid tumors (17, 18). In melanoma, NEDD9 interacts with focal adhesion kinase (FAK) to promote cell invasion and metastasis (19). Similar observation has been reported in...
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glioblastoma studies (20). These data support an important role of NEDD9 in cancer progression and metastasis of a number of malignancies. However, the interactive regulation between LKB1 and NEDD9, and the potential functional contribution of such a network to lung cancer progression and metastasis has not been explored yet.

Here, we provide strong evidences for NEDD9 as an important downstream mediator in LKB1-deficient lung cancer progression, and uncover a novel mechanism of LKB1 in contribution to lung cancer progression through CRTC1-NEDD9 axis. Our data provide novel functional evidences and mechanistic insights into lung cancer progression and metastasis evoked by LKB1 loss and define a potential biomarker for lung cancer prognosis in clinic.

Materials and Methods

Mouse colony, mouse treatment, and mouse tumor analyses

KrasG12D and Lkb1L/L mice were generously provided by Drs. T. Jacks and R. Depinho, respectively (4). Nude mice (6 weeks old, male) were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. All mice were housed in a specific pathogen-free environment at Shanghai Institute of Biochemistry and Cell Biology and treated in strict accordance with protocols approved by the Institutional Animal Use Committee of the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

The KrasG12D or KrasG12D, Lkb1L/L mice at 6 to 8 weeks old were virally infected with either 2 × 10⁸ PFU Adeno-Cre or lentivirus (Lenti-Cre, NEDD9-Cre, Ctrl-Cre, or shNedd9-Cre) via nasal inhalation as previously described (21, 22). The lentiviruses were prepared as previously described (4). All mice were sacrificed for gross inspection and pathologic examination. Lung tumors were dissected for molecular analyses. The number of total tumor, well- or poorly differentiated tumors as well as the total tumor area were analyzed for each group from sectioned H&E slides as described before (23, 24) and the data were presented as mean ± SEM. As for lung seeding assay, nude mice were injected with 1 × 10⁶ cells via tail veins. After 10 weeks of inoculation, all mice were sacrificed and mouse lungs were weighted and lung metastasis was detected by H&E staining from series section.

Human lung cancer specimen analyses

All 175 human lung cancer samples were collected with patient consents in Fudan University Shanghai Cancer Center (Shanghai, China) from October 2007 to February 2008 with approval from the Institute Research Ethics Committee.

Cell culture, plasmids, and antibodies

Plasmids construction and the detailed information for antibodies are described in Supplementary Materials and Methods. Human cell lines including A549, CRL-3907, CRL-5866, HEK-293, and HEK-293T were purchased from American Type Culture Collection (ATCC) and authenticated by ATCC by short tandem repeat (STR) profiling and used for functional studies within 6 months after thaw from liquid nitrogen tank. A549, CRL-3907, CRL-5866 and HEK-293, and HEK-293T cells were, respectively, cultured in RPMI-1640 medium (HyClone) or Dulbecco’s Modified Eagle’s Media (HyClone) supplemented with 10% FBS (Biochrom AG) and antibiotics (100 U/mL streptomycin and 100 μg/mL penicillin; Invitrogen).

Immunohistochemical analysis

Immunohistochemistry was conducted as previously described (24). The proliferation rate was assessed by counting Ki-67 positive nuclear staining at high-power field for more than 1,000 cells. The apoptosis was assessed by analysis of cleaved caspase 3 immunostaining. The NEDD9 immunostaining was reviewed and scored blindly into 2 criteria as low and high expression as previously described (25). The correlation between clinical features and NEDD9 expression in human lung cancer was analyzed by SPSS 13.0 statistical software, and P values were calculated by Pearson χ² test. A value of P < 0.05 was considered as significant (2 tailed).

RT-PCR, real-time RT-PCR, and Western blot analysis

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions and retrotranscribed into cDNA (Ferments). [Primers for reverse transcriptase (RT-PCR) and real-time RT-PCR are listed in Supplementary Table S1]. Western blot analysis was done as previously described (26). The samples of cytoplasm and nuclear protein were obtained using proteoextract subcellular extraction regents (Calbiochem) according to the manufacturer’s instruction.

Reporter gene assay

Luciferase activities were measured 48 hours after transfection using the Dual-Luciferase Assay kit (Promega) on a GloMax 20/20 luminometer (Promega). pRL-SV40 was cotransfected as internal control. Experiments were conducted in triplicates and repeated at least 3 times.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was conducted as previously described (27). Briefly, 5 μg antibody of rabbit anti-cAMP-responsive element binding protein (CREB) or rabbit anti-CRTC1 was added into each lytic sample at 4°C overnight. Rabbit IgG was used as negative control. Fifty microliter Protein A (Invitrogen) was added into each sample for immunoprecipitation, and then samples were rotated at 4°C for 2 hours. An aliquot of sonicated cleared extract (input) and the immunoprecipitated material were decross-linked in TE plus 1% SDS for at least 8 hours at 65°C. Primers for ChIP assay were listed in Supplementary Table S1. NRR4A2 and GAPDH served as positive and negative controls, respectively (28). Representative data were shown from 3 independent experiments.

Wound healing assay

Wound healing assay was conducted as described before (29). Cells at 100% confluence were starved in serum-free medium for more than 12 hours dependent on different cell lines. Cells were then lightly and quickly scratched with a
pipette tip. The cell migration kinetics were closely monitored, photographed, and calculated by IncuCyte 2008B.

Three-dimensional cell culture
Cells were seeded in medium contain 2% Matrigel (BD Biosciences) on the top of another layer of solidified Matrigel. Cells were then cultured at 37°C incubators and monitored for the appearing morphologic changes in 1 to 2 weeks. Photos were taken using a light microscope (Leica).

Statistical analysis
Data were analyzed by Student t test. P < 0.05 was considered to be significant (2 tailed). Error bars represent SEM.

Results
Transcriptional regulation of NEDD9 by LKB1 in mouse and human lung tumors
Our previous gene expression profiling analyses of mouse Kras<sup>G12D</sup> lung tumors with and without Lkb1 deficiency identified Nedd9 as a potential target gene downstream of Lkb1 (4). To extend this finding, we investigated the functional correlation of NEDD9 and LKB1 in human lung cancer. We found that knockdown of LKB1 in human NSCLC cell lines CRL-5866 and CRL-5907, which express wild-type LKB1, resulted in increased NEDD9 mRNA and protein levels (Fig. 1A–F). Reciprocally, ectopic LKB1 expression in the LKB1-mutant A549 human NSCLC cells inhibited NEDD9 expression (Fig. 1G). The regulation was associated with marked induction of Nedd9 expression in Lkb1 deficient mouse tumors in vivo (Fig. 1H and I). These data show that LKB1 is a negative regulator of NEDD9 transcription in mouse and human lung tumors.

NEDD9 is an important downstream mediator of lung cancer progression and metastasis evoked by Lkb1 deficiency
We next investigated the functional interaction between NEDD9 and LKB1 in lung cancer progression and metastasis. We found that LKB1 overexpression in A549 cells blocked colony formation in soft agar, migration in wound healing assays, invasion in Matrigel as well as in boyden chamber assay whereas these effects were reversed by ectopic NEDD9 expression (Fig. 2A–E and Supplementary Fig. S1). Similarly, the


inhibition of tumor formation by LKB1 overexpression in A549 lung seeding assay was partially overcome by ectopic NEDD9 expression; large nodules were detectable in all mice from the control group, in 1/8 mice from the LKB1 group, and in 4/8 mice from the LKB1/NEDD9 group (Fig. 2F and G). These data showed that NEDD9 can partially rescue the inhibitory function of LKB1 upon lung cancer invasion and metastasis.

Nedd9 knockdown inhibits de novo lung cancer progression in Kras⁶¹¹²⁰, Lkb¹⁻⁻ mice model

We next asked if Nedd9 knockdown affects de novo lung cancer progression (4). We infected Kras⁶¹¹²⁰, Lkb¹⁻⁻ mice with lentivirus carrying Cre expression (Ctrl-Cre) or Cre expression together with Nedd9 knockdown (shNedd9-Cre) as previously described (21). Mice were sacrificed for gross inspection and pathologic analyses at 21 weeks post viral administration. Knockdown of Nedd9, confirmed by real-time RT-PCR and immunostaining analyses (Supplementary Fig. S2A and S2B), resulted in a slight decrease of the number of tumors on lung surface but not total tumor number and area (Supplementary Fig. S2C–S2E). More importantly, we found that the percentage of poorly differentiated tumors significantly decreased in Nedd9-knockdown group in comparison with control group (Fig. 3A and B). Consistently, Nedd9 knockdown also resulted in a decrease of cell proliferation rate (Fig. 3C and D) while had no effect on cell apoptosis (Supplementary Fig. S2F). These data further support the essential role of Nedd9 in regulating de novo lung cancer progression in context with Lkb1 deficiency.

Ectopic NEDD9 expression promotes de novo lung cancer progression in Kras⁶¹¹²⁰ mouse model

We further investigated whether ectopic Nedd9 expression is sufficient to promote lung cancer progression in de novo Kras⁶¹¹²⁰ mouse model. We treated Kras⁶¹¹²⁰ mice with lentivirus carrying either Cre (Lenti-Cre) or Cre with NEDD9 expression (NEDD9-Cre) via nasal inhalation and all mice were sacrificed for analyses at 24 weeks post viral administration. We found that ectopic NEDD9 expression, confirmed by real-time RT-PCR and immunostaining analyses (Supplementary Fig. S3A and S3B), did not affect the total number and area of
We found that ectopic expression of CREB and/or transcription activity of CREB and its coactivator CRTC1, consistent with the inhibitory role of LKB1 upon the knockdown in CRL-5866 cells. Interestingly, CRE reporter like promoter (TAL), and AP1 for their response to LKB1 response element (GRE), heat-shock element (HSE), TATA-box, CRE, serum response element (SRE), NF-κB, glucocorticoid response element (GRE), heat-shock element (HSE), TATA-like promoter (TAL), and API for their response to LKB1 knockdown in CRL-5866 cells. Interestingly, CRE reporter was the only one regulated by LKB1 (Supplementary Fig. S4), consistent with the inhibitory role of LKB1 upon the transcription activity of CREB and its coactivator CRTC1 (30, 31). We found that ectopic expression of CREB and/or CRTC1 significantly upregulated NEDD9 mRNA and protein levels (Fig. 5A and B). Conversely, knockdown of CRTC1 dramatically decreased the NEDD9 mRNA level (Supplementary Fig. S5). To further explore the regulation of NEDD9 by CRTC1/CREB, we cloned the 3kb genomic DNA fragment containing NEDD9 promoter for reporter gene assays. Through a series of deletion/mutation analyses (Supplementary Fig. S6), we had narrowed down the CRTC1/CREB -responsive element to a 20bp fragment (−146 to −166) with a nonclassical CRE site “TGAGCTCA” (32), which could not be predicted by TFSCAN (http://www-bimas.cit.nih.gov/molbio/proscan; ref. 33). Mutation of this non-classical CRE site abolished the response of NEDD9 promoter to either CREB or CRTC1 expression (Fig. 5C and D). Results from ChIP assay confirmed the de novo binding of either CREB or CRTC1 to NEDD9 promoter region (Fig. 5E). These data convincingly show that CRTC1/CREB participates in regulating NEDD9 gene transcription.

Moreover, we found that LKB1 knockdown or ectopic LKB1 expression regulated the transcriptional activity of NEDD9 promoter with wild-type nonclassical CRE site but not with mutated site (Supplementary Fig. S7). Ectopic CRTC1 expression partially reversed the decrease of NEDD9 mRNA and protein levels caused by ectopic LKB1 expression in A549 cells (Fig. 5F and G). Immunofluorescence analysis showed that ectopic LKB1 expression resulted in CRTC1 subcellular localization of LKB1, whereas more nodules were observed on lung surface from NEDD9-Cre group than control (Supplementary Fig. S3C–S3E). Detailed pathologic analyses further revealed that the percentage of poorly differentiated tumors was significantly higher in NEDD9-Cre group (Fig. 4A and B). Consistently, a higher proliferation rate was observed in lung tumors with ectopic NEDD9 expression (Fig. 4C and D). However, no significant change of apoptosis was found in NEDD9 overexpressed lung tumors (Supplementary Fig. S3F). Taken together, these data show that ectopic NEDD9 expression significantly promotes lung cancer progression in de novo mouse model.

**CRTC1 regulates NEDD9 transcription and contributes to LKB1-deficient lung tumor progression**

To determine how LKB1 negatively controls NEDD9 expression, we initially screened seven reporters including CRE, serum response element (SRE), NF-κB, glucocorticoid response element (GRE), heat-shock element (HSE), TATA-like promoter (TAL), and API for their response to LKB1 knockdown in CRL-5866 cells. Interestingly, CRE reporter was the only one regulated by LKB1 (Supplementary Fig. S4), consistent with the inhibitory role of LKB1 upon the transcription activity of CREB and its coactivator CRTC1 (30, 31). We found that ectopic expression of CREB and/or...
translocation from nucleus to cytoplasm (Fig. 5H). This was further confirmed by Western blot analysis using cytoplasmic and nuclear fractions (Fig. 5I). Previous studies have shown that salt inducible kinase (SIK), a downstream substrate of LKB1, is important in linking LKB1 and CRTC1/CREB transcription activity (31). Our data showed that ectopic LKB1 expression induced nuclear translocation of SIK2, which results in CRTC1 nuclear exportation in human lung cancer cells (Supplementary Fig. S8). This regulation was further confirmed by LKB1 knockdown experiment (Supplementary Fig. S9). Our data showed that ectopic LKB1 expression induced nuclear translocation of SIK2, which results in CRTC1 nuclear exportation in human lung cancer cells (Supplementary Fig. S8). This regulation was further confirmed by LKB1 knockdown experiment (Supplementary Fig. S9). Moreover, SIK2 knockdown resulted in CRTC1 nuclear retention which eventually upregulates NEDD9 transcript (Supplementary Fig. S10). These data convincingly showed that LKB1 regulates NEDD9 gene expression through CRTC1 cellular translocation via SIK2 in lung cancer.

We further found that ectopic CRTC1 expression partially rescued the inhibition of colony formation, cell migration, and invasion caused by LKB1 expression in A549 cells (Fig. 6A–D and Supplementary Fig. S11), as well as the inhibitory effect of LKB1 upon lung cancer metastasis in lung seeding assay (Fig. 6E and F). Taken together, these data show that CRTC1 regulates NEDD9 gene expression and contributes to LKB1-deficient lung tumor progression.

High NEDD9 expression correlates with human lung cancer malignancy progression and metastasis

Finally, we examined the clinical relevance of NEDD9 expression in human lung cancer. A cohort of 175 human NSCLC samples was used for NEDD9 immunostaining (Supplementary Fig. S12). Remarkably, high NEDD9 expression was strongly correlated with lymph node metastasis and advanced clinical stage, as well as with smoking and low differentiation (Table 1). No significant correlations were observed between NEDD9 expression and other clinical features, including NSCLC subtypes and tumor size (Table 1). Therefore, high NEDD9 level appears to be an indicator of poor prognosis for human lung cancer.
Discussion

Metastasis is a major factor contributing to the high mortality of lung cancer. Here, we discover an important novel pathway involved in lung cancer progression and metastasis triggered by \textit{LKB1} loss: \textit{LKB1} loss triggers the nuclear translocation of CRTC1, which in turn upregulates NEDD9 and promotes lung cancer progression and metastasis. Our study highlights NEDD9 as an important downstream mediator in lung cancer progression evoked by \textit{LKB1} loss. High NEDD9 expression strongly associates with poor differentiation, advanced clinical stage, as well as lymph node metastasis. Collectively, these data provide a novel mechanism by which \textit{LKB1} loss promotes cancer progression and metastasis as well as the potential biomarker for human NSCLC prognosis.

Our previous work showed that NEDD9 was upregulated by \textit{LKB1} loss in lung cancer metastasis but did not resolve how NEDD9 potentially links \textit{LKB1} loss to the malignant phenotype (4). Recent studies have shown that activation of hypoxia-inducible factor-1α (HIF-1α), downstream of mTOR pathway, regulates NEDD9 expression in colon, and renal cancers (34, 35). We have previously shown that the mTOR-HIF-1α axis actually upregulates LOX, which in turn cross-links collagen to remodel extracellular matrix and triggers β1-integrin signaling through FAK activation, and thus promotes lung cancer metastasis (11). However, no evidence of the involvement of mTOR activation was found in NEDD9 regulation (4). Interestingly, CRTC1/CREB transcription activation is critical for mediating NEDD9 expression and potentially promotes lung tumor malignancy. Nuclear translocation of CRTC1 regulated by \textit{LKB1} loss promotes NEDD9 gene expression in lung cancer, which may indicate a cell type–specific pattern of \textit{NEDD9} gene regulation. As a coactivator of CREB, CRTC1 forms complex with CREB in nucleus and turns on the CREB transcriptional activity (30, 31, 36), which plays important roles in glucose and lipid metabolism (31, 37–39). However, apart from its role in metabolic regulation, less is known about its potential contribution in lung cancerogenesis. Previous studies have identified SIK as an important mediator of CRTC1/CREB transcription activity downstream of \textit{LKB1} (31). In \textit{LKB1}-deficient cells, SIK is incapable of phosphorylating CRTC1, resulting in CRTC1 nucleus retention where it interacts with CREB and promotes certain gene expression (30, 31, 36, 40, 41). Previous study has shown that NR4A2 is transcriptionally upregulated by CRTC1/CREB pathway in \textit{LKB1} null lung cancer cells and promotes cell growth (42). Our data here identify NEDD9 as another important downstream mediator of CRTC1/CREB pathway in lung cancer progression and metastasis caused by \textit{LKB1} loss. Further dissection of this
circuitry may provide important hints for targeted therapy in \textit{LKB1}-deficient lung cancer.

With integrative studies using \textit{de novo} animal model, lung seeding assay as well as \textit{in vitro} migration and invasion systems, we have convincingly shown that NEDD9 plays a positive role in lung cancer progression and metastasis. Previous studies have supported that NEDD9 promotes solid tumor metastasis via FAK and Src activation (19, 20, 43) through direct interaction (18). Interestingly, 2 recent studies have implicated the potential involvement of NEDD9 in epithelial–mesenchymal transition (EMT) process in breast cancer (44, 45). EMT is thought to be an important event involved in early stages of cancer metastasis (46). It is attempting to ask if Nedd9 mediates the EMT process observed in \textit{Lkb1}-deficient lung cancer progression and metastasis (12).

We further investigated the clinical correlation of NEDD9 expression level in 175 lung cancer specimens. Interestingly, we have found that high NEDD9 expression is positively correlated with lymph node metastasis and malignant progression, suggesting that NEDD9 is an indicator for poor prognosis of lung cancer patients. Future studies with an even larger sample number will be needed to establish a correlation between NEDD9 expression and survival.

In summary, our work has provided mechanistic insights into lung cancer progression and metastasis evoked by \textit{LKB1} loss. NEDD9 also serves as a potential biomarker for lung cancer prognosis.

\textbf{Disclosure of Potential Conflicts of Interest}

K.-K. Wong has a commercial research grant from AstraZenca and Millenium, and is a consultant/advisory board member of Molecular MD. No potential conflicts of interest were disclosed by the other authors.

\textbf{Authors’ Contributions}

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Feng, Z. Fang, N. Bardeesy, K.-K. Wong, H. Ji
Writing, review, and/or revision of the manuscript: Y. Feng, Y. Gao, T. Xiao, F. Li, N. Bardeesy, K.-K. Wong, H. Ji
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