

RESEARCH ARTICLE

Lentivirus-mediated shRNA Interference Targeting SLUG Inhibits Lung Cancer Growth and Metastasis

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Abstract

Objective: Lung cancer is a deadly cancer, whose kills more people worldwide than any other malignancy. SLUG (SNAI2, Snail2) is involved in the epithelial mesenchymal transition in physiological and in pathological contexts and is implicated in the development and progression of lung cancer. **Methods:** We constructed a lentivirus vector with SLUG shRNA (LV-shSLUG). LV-shSLUG and a control lentivirus were infected into the non-small cell lung cancer cell A549 and real-time PCR, Western blot and IHC were applied to assess expression of the SLUG gene. Cell proliferation and migration were detected using MTT and clony formation methods. **Results:** Real-time PCR, Western Blot and IHC results confirmed down-regulation of SLUG expression by its shRNA by about 80%~90% at both the mRNA and protein levels. Knockdown of SLUG significantly suppressed lung cancer cell proliferation. Furthermore, knockdown of SLUG significantly inhibited lung cancer cell invasion and metastasis. Finally, knockdown of SLUG induced the down-regulation of Bcl-2 and up-regulation of E-cadherin. **Conclusion:** These results indicate that SLUG is a newly identified gene associated with lung cancer growth and metastasis. SLUG may serve as a new therapeutic target for the treatment of lung cancer in the future.

Keywords: SLUG - lung cancer - proliferation - apoptosis-metastasis

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Introduction

Lung cancer is the most common diagnosed cancer in the world and the most common cause of the cancer mortality worldwide. The high mortality is largely due to the late stage of diagnosis and the poor response to therapy. The need to develop better diagnostic techniques and therapies is urgent (Tsoi et al., 2012). Although substantial advances have been made in lung cancer research (Malapelle et al., 2012), survival to incidence ratio is still poor and overall cure rate remains very low (Ravenel, 2012). Therefore, studying the mechanism of tumor invasion and metastasis will provide further insights into the development and progression of lung cancer.

In lung cancer, SLUG confers resistance to the epidermal growth factor receptor tyrosine kinase inhibitor (Chang et al., 2011). SLUG, a member of the snail family of transcription factors, plays a crucial role in regulation of epithelial-mesenchymal transition by suppressing several epithelial markers and adhesion molecules (Nieto, 2002; Barrallo-Gimeno et al., 2009). Knockdown of SLUG could effectively sensitize the cells to the stimulators above through PUMA upregulation (Vannini et al., 2007; Vitali et al., 2008; Zhang et al., 2011; Zhang et al., 2011). Except for PUMA, SLUG down-regulation facilitates apoptosis induced by proapoptotic drugs in neuroblastoma cells by downregulation of Bcl-2 prototypic antiapoptotic protein

(Vitali et al., 2008).

Small hairpin RNA (shRNA) expression vector systems have been established to induce RNA interference (RNAi) in mammalian cells (Yang et al., 2012). Although these vectors provide certain advantages over chemically synthesized siRNAs, some disadvantages remain, including transient shRNA expression and low transfection efficiency, especially in non-dividing primary cells. To overcome these limitations, shRNA delivery systems using retroviral vectors (Araki et al., 2012), adenoviral vectors (El-Armouche et al., 2007) and, more recently, lentiviral vectors (Hung et al., 2010) have been reported and proven to be safe for humans. Lentivirus vectors encoding antisense targeting sequence have been used in clinical trials with no obvious side effects (Manilla et al., 2005). Lentivirus-delivered shRNAs are capable of specific, highly stable and functional silencing of gene expression in a variety of human cells including primary nondividing cells and also in transgenic mice (Rubinson et al., 2003).

In our study, we constructed a lentivirus vector mediating RNAi targeting of SLUG (LV-shSLUG). The efficacy of LV-shSLUG plasmids in interference with SLUG was confirmed by real-time PCR and western blot. Furthermore, the present in vitro findings suggest that the down-regulation of SLUG may represent a novel targeted therapy for lung cancer.

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Materials and Methods

Cell lines

A549 and HEK293T cell lines were obtained from Shanghai Institute for Biological Sciences, Chinese Academy of Sciences and maintained in RPMI-1640 supplemented with 10% fetal calf serum and antibiotics, and frozen down to maintain limited passage history.

RNA interference

Self-inactivating lentivirus vector (GeneChem, Shanghai, China) containing a CMV-driven GFP reporter and a U6 promoter upstream of the cloning sites (Age I and EcoR I) was used for cloning small hairpin RNAs (shRNAs). The target sequence for SLUG was 5'-GGAATATGTGAGCCTGGGCGCC-3'; the negative control sequence was 5'-GAACCGTGTCTTCCTCAGTATC-3'. The A549 cell line was cultured in six-well tissue culture plates and infected with lentivirus at a multiplicity of infection (MOI) of 10 for 24 h. Then the medium was replaced with fresh complete medium. After 4 days, cells were observed under fluorescence microscopy to confirm that more than 80% of cells were GFP-positive.

Real-time quantitative RT-PCR (q-RT-PCR)

Total RNA was extracted using Trizol reagent (TaKaRa) and reversed transcribed. Quantitative real-time PCR analysis was performed using LightCycler 480 (Roche). Real-Time PCR System: Each well (20 μ L reaction volume) contained 10 μ L Power SYBR Green PCR master mix (TaKaRa), 1 μ L of each primer (5 μ mol/L) and 1 μ L template. The following primers were used: SLUG sense 5'-GACACACATACAGTGATTATT-3'; and antisense 5'-AAACTTTTCAGCTTCAATGGC-3', GAPDH sense 5'-CCCTTCATTGACCTCAACTA-3'; and antisense 5'-CCAAAGTTGTTCATGGATGAC-3'.

Western blot

Cells were lysed in RIPA buffer containing 1mM PMSF. Forty microgramme of protein per lane was resolved by SDS-PAGE and transferred to PVDF membrane and blocked with 5% BSA in PBST. After incubating with primary antibody rabbit anti-human SLUG (ab27568, Abcam, Cambridge, MA), rabbit anti-human-Bcl-2 (ab7973), rabbit anti-human-E-cadherin (ab15148) or rabbit anti-human-GAPDH (ab9485) overnight at 4 °C and secondary antibody horseradish peroxidase-conjugated anti-rabbit IgG for 1 h at room temperature, blots were developed using ECL method.

Immunohistochemistry (IHC)

Logarithmic growth phase cells were seeded in 6-well plate covered with coverslips inside, after 24 h culture, coverslips were collected and stained by immunohistochemistry (IHC). IHC was performed according to standard protocols. The primary antibody was rabbit anti-human SLUG polyclonal antibody (ab27568, Abcam, Cambridge, MA) diluted in PBS with 0.1% TritonX-100.

Cell proliferation assays

Cell proliferation was determined by using MTT assay and anchorage independent soft agar colony formation assay. For MTT assay, 20 μ L MTT (5 mg/ml in PBS) was added directly into each well of 96-well plate and incubated at 37 °C for 4 h. Then media was removed and 200 μ L DMSO was added to dissolve formazan crystals. The optical density at 570 nm was read with a microplate reader (Molecular Devices, Sunnyvale, CA). For soft agar colony formation assay, 500 μ L 2 \times DMEM supplemented with 20% FBS was mixed with 500 μ L 1.2% Sea Plaque agar and solidified in each well of a 24-well plate to form base agar layer. For top agar layer, 25 μ L cells (5×10^3 /ml) were mixed with 250 μ L 2 \times DMEM and 500 μ L 0.7% Sea Plaque agar and added on top of base agar layer. After grown for 14 days, colony formation was monitored under microscopy. A cluster of ten cells or more was defined as a colony.

Colony formation in soft agar

To assess anchorage-independent growth, soft agar clonogenic assays were done. Each well of a 6-well plate contained 2 mL of 0.5% (w/v) Noble agar (Difco) in DMEM with 10% NBCS. Cells, (3×10^3) in 2 mL of 0.375% (w/v) Noble agar in 10% NBCS DMEM were added above the polymerized base solution. All solutions were kept at 40 °C before pouring to prevent premature agar polymerization and to ensure cell survival. Plates were incubated (37 °C, 5% CO₂) under standard conditions for 10 days before colony number and diameter were quantified microscopically.

Cell migration assay

Invasion assay was performed as described previously (Albini et al., 1987). Cell migration assay was also performed using Boyden chambers. Cells (2×10^5) were resuspended in 200 μ L serum-free media and seeded into the upper chamber. Conditioned media of A549 cell culture was filtered and added to the lower chamber as a chemotactic factor. After 24 h, non-migrating cells remaining on the upper surface were removed, and cells on the lower surface were fixed, stained with crystal violet, and counted. Each experiment was performed at least in triplicate and the data were expressed as mean \pm SD.

Statistical analysis

The results are expressed as the mean \pm SD. For comparison of means between two groups, a two-tailed t-test was used and for comparison of means among three groups, one-way ANOVA were used. Statistical analysis was performed using SPSS software version 13.0. P-value < 0.05 was considered statistically significant.

Results

Knockdown of SLUG expression by lentivirus-mediated RNA interference

To investigate the role of SLUG in lung cancer, we constructed lentivirus vector with SLUG shRNA and infected the A549 cell line. After viral infection, more than

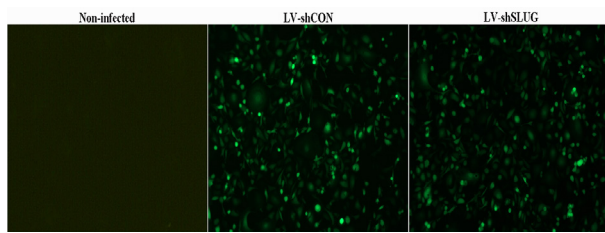


Figure 1. Verification of SLUG Knockdown in A549 Cell Line by Lentivirus-mediated RNA Interference. GFP expression images showing shRNA delivery efficiency. (Magnification $\times 200$)

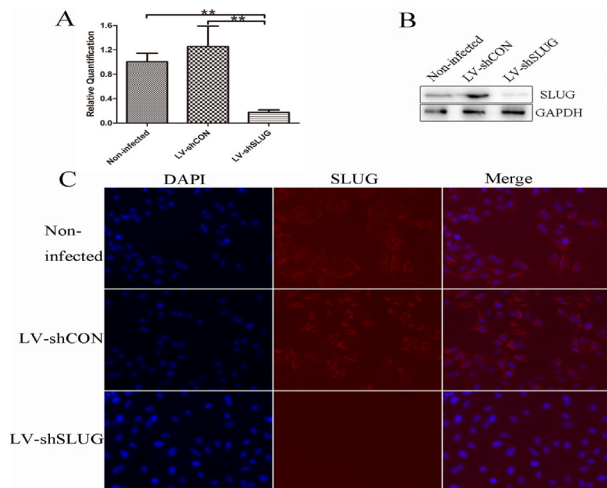


Figure 2. Verification of Knockdown of SLUG Expression in A549 by Lentivirus-mediated RNA Interference. (A) SLUG mRNA expressions in lentivirus-infected cells as measured by Real-time PCR. (B) SLUG protein expressions in lentivirus-infected cells as measured by Western blot. (C) SLUG protein expression in lentivirus-infected cells as measured by IHC staining (Magnification $\times 200$). $**P < 0.05$ versus control

80% cells were GFP-positive, indicating a high efficiency of shRNA delivery (Figure 1). Real-time RT-PCR, Western Blot and IHC confirmed the down-regulation of SLUG expression by its shRNA. As shown in Figure 2, SLUG expression was reduced by about 80%~90% in the A549 cells at both the mRNA and protein levels after RNA interference. The immunohistochemical staining of SLUG was detected mainly in the cytoplasm.

Knockdown of SLUG expression suppressed lung cancer cell proliferation

MTT results showed that SLUG knockdown significantly reduced cell proliferation of A549 (Figure 3A). In soft agar colony formation assay, A549 cells infected with SLUG shRNA showed significant reduction in the colony formation (Figure 3B&C). No significant difference was found between control shRNA infected cells and non-infected cells.

SLUG silencing of A549 cells expression inhibited lung cancer cell migration

As shown in Figure 4A, the average cell migrating count of SLUG shRNA infected cells was much less than that of control shRNA infected cells. As shown in Figure 4B, the average migrating cell count of SLUG shRNA infected cells was much less than that of control

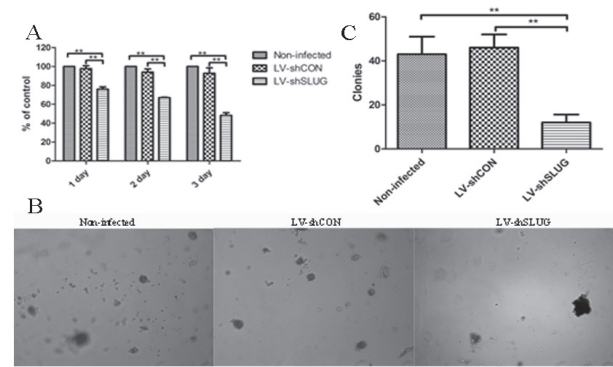


Figure 3. Effect of SLUG Knockdown in Invasive Cell Line A549 on Cell Growth and Colony Formation. (A) Cell proliferation as examined by MTT assay. (B, C) Anchorage independent growth as measured by soft agar colony formation assay. (Magnification $\times 100$). $**P < 0.05$ versus control

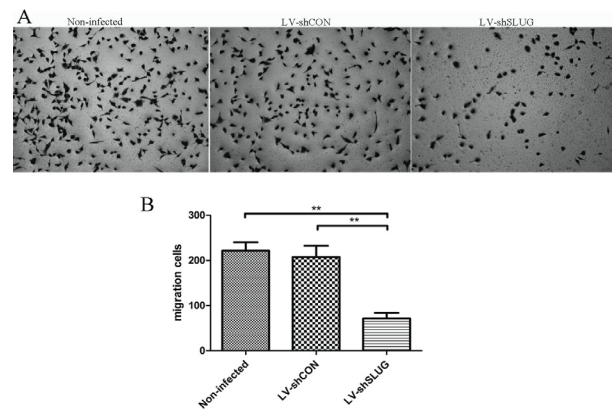


Figure 4. Effect of SLUG Knockdown in Highly A549 Cell Line on Cell Migration. (A&B) Cell migration assay using Boyden chambers. (Magnification $\times 200$). $**P < 0.05$ versus control

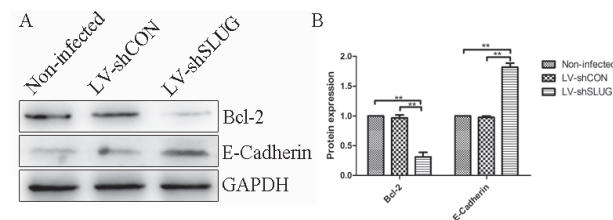


Figure 5. Bcl-2 and E-cadherin Immunodetection in LV-shSLUG, shCON and Non-infected A549 Cells. (A) Cell extracts were analyzed for expression of the indicated proteins using specific antibodies. The experiment was repeated twice with similar results. (B) Expression level of each protein in controls was set to 1.0. Levels are reported as percent of control \pm SD. $**P < 0.05$ versus control

shRNA infected cells. No significant difference was found between control shRNA infected cells and non-infected cells.

Knockdown of SLUG expression in A549 cells modulates bcl-2 and E-cadherin expression

It has been demonstrated that SLUG is involved in the epithelial-to-mesenchymal transition (EMT), linked to the acquisition of the invasive phenotype (Miyake et al., 2001), we analyzed the expression of several Slug targets in LV-shSLUG-transfected A549 cells by western blot. In LV-shSLUG-transfected A549 cells, anti-apoptotic Bcl-2 was down-regulated (Figure 5 A&B, $P < 0.05$). E-cadherin,

a well-known target of SLUG previously described (Miyake et al., 2001), was up-regulated in LV-shSLUG-transfected cells (Figure 5 A&B, $P < 0.05$). There were no different expressions of Bcl-2 and E-cadherin between non-infected and LV-shCON infected A549 cells.

Discussion

Worldwide, lung cancer to be leading cause of death in men and women (Archontogeorgis et al., 2012). Cancer metastasis and resistance to treatment (including radiotherapy, chemotherapy and targeted therapy) are two major causes for the poor survival of lung cancer patients. Indeed, it is now to discover the mechanism of tumor metastasis and resistance. Events frequently observed in the malignant transformation of epithelial cells include the loss of epithelial differentiation, a decrease in cell-cell contact, and the acquisition of invasive and migratory properties. Epithelial-mesenchymal transition (EMT) is involved in cancer cell invasion, resistance to apoptosis and stem cell features (Talbot et al., 2012).

The process of EMT is controlled by a group of transcriptional factors, zinc finger proteins and basic helix-loop-helix factors. In this regard, the members of the Snail family of zinc-finger proteins, SLUG, have been found to play a central role in this phenomenon referred to as epithelial-to-mesenchymal transition (Nieto, 2002). Recently, another critical role of SLUG has been reported. SLUG binds to E-box elements in the proximal E-cadherin promoter and represses transcription of the E-cadherin gene (Ma et al., 2007). Experimental data have led to the inclusion of Slug into the SLUG of transcription regulators involved in tumor progression and metastasis (Peinado et al., 2007). Previous data suggest that SLUG expression significantly correlated with reduced E-cadherin expression in patients with esophageal squamous cell carcinoma, and patients with reduced E-cadherin expression or positive SLUG expression have a poor clinical outcome (Uchikado et al., 2005).

In functional assays, we constructed lentivirus vector with SLUG shRNA and infected the A549 cell line. Using MTT assay, soft agar colony formation assay, we concluded that knockdown of SLUG suppressed lung cancer cell proliferation. In further study, we found that knockdown of SLUG could decrease the expressions of Bcl-2 which suggested that SLUG could play an effect on proliferation via controlling the expressions of the cell cycle proteins. Slug binds to E-box elements in the proximal E-cadherin promoter and represses transcription of the E-cadherin gene (Ma et al., 2007). Experimental data have led to the inclusion of SLUG into the Snail family of transcription regulators involved in tumor progression and metastasis (Peinado et al., 2007).

In conclusion, we identified SLUG is a newly identified gene associated with lung cancer growth and metastasis. Knockdown of SLUG expression significantly suppressed lung cancer cell proliferation, induced cell apoptosis and inhibited cell invasion and metastasis. SLUG may serve as a potential therapeutic target for the treatment of lung cancer in the future.

Acknowledgements

The author(s) declare that they have no competing interests.

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