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Effect of Slug-Mediated Down-Regulation of E-Cadherin on Invasiveness and Metastasis of Anaplastic Thyroid Cancer Cells

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Manuscript Preparation E
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Background: Slug has been found to promote migration and invasion of many cancer cells, including anaplastic thyroid cancer (ATC). Thus, targeting Slug expression could provide new approaches for the treatment of patients with ATC.

Material/Methods: Small interfering RNA (siRNA) targeting Slug (Slug siRNA) was used to construct clonal derivatives in the metastatic ATC SW1736 cells. Slug cDNA transfection was used to restore the Slug expression in the Slug siRNA-transfected SW1736 cells (Slug siRNA/SW1736). E-cadherin siRNA was used to inhibit E-cadherin expression in the Slug siRNA/SW1736 cells. The SW1736 cell migration, invasion, and signaling pathway was analyzed *in vitro*.





Furthermore, the stable Slug siRNA-transfected SW1736 clones were used for the lung metastasis assay in an *in vivo* mouse model.

Results: Targeting Slug expression in SW1736 cells showed a 45% decrease in migration and an 85% decrease in invasiveness *in vitro*. Knockdown of E-cadherin by E-cadherin siRNA transfection or Slug overexpression by Slug cDNA transfection restored the invasive and migrative ability in SW1736 cells. In addition, we found an 80% decrease in the number of macroscopic lung metastases nodes of mice by *in vivo* analysis. Western blot assay showed that Slug expression was inhibited and E-cadherin expression was increased in the Slug siRNA-transfected tumors.

Conclusions: Targeting Slug signaling pathway is effective in preventing lung metastasis in ATC.

MeSH Keywords: **Cadherins • Head and Neck Neoplasms • Neoplasm Metastasis**

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Background

Anaplastic thyroid cancer (ATC) accounts for 1–2% of all thyroid tumors. It is characterized by aggressive, local invasion and common distant metastases. ATC is histologically characterized by elevated mitotic rate and lymphovascular invasion [1]. The epithelial to mesenchymal transition (EMT) is an important factor contributing to the invasiveness of ATC [2,3]. Down-regulation of epithelial markers (E-cadherin) can induce the loss of cell-cell adhesion, cell polarity, and result in migratory and invasive properties [4,5]. Upregulation of E-cadherin expression is a new method for the prevention of metastasis in ATC.

Slug (Snail2) is a zinc finger transcriptional repressor. It is conserved among vertebrate species, mediating sequence-specific interactions with DNA [6,7]. Slug has been implicated in multiple cellular processes, many of which relate to cell motility and induction of the EMT. Additionally, Slug is aberrantly expressed in various

cancers, in which they regulate cell invasion, metastasis, cell survival, and proliferation [8]. Recently, it was found that Slug is not expressed in normal thyroid cells. However, Slug was found to be highly expressed in thyroid carcinoma cell lines and metastatic thyroid carcinoma samples [9]. Buehler et al. has found that Slug was negative in follicular adenomas, lower in papillary and follicular thyroid carcinomas, but positive in the ATCs or cell line. This suggests that Slug plays an important role in EMT in the development of ATC [10].

We previously described an important role of Slug in the invasiveness and metastasis of extrahepatic hilar cholangiocarcinoma [11], esophageal adenocarcinoma [12], bladder cancer [13], and pancreatic cancer [14]. Anaplastic thyroid carcinoma (ATC) is one of the most aggressive human cancers and often shows resistance to multimodal therapeutic approaches. A previous study reported that knockdown of Slug inhibited growth and enhanced the antitumor activity of DOX in ATC cells via induction of PUMA upregulation, indicating that Slug has good potential for the development of new therapeutic strategies for treating ATC [15]. However, it remains to be addressed whether and how Slug affects the cell invasiveness and migration of ATC cells.

In the present study, we investigated the effect of targeting Slug or Slug overexpression on invasiveness and metastasis of ATC cells *in vitro* and *in vivo*. Our results indicated Slug promotes *in vitro* invasiveness by down-regulation of E-cadherin. Knockdown of Slug inhibits *in vitro* and *in vivo* invasiveness and metastasis by upregulation of E-cadherin. We therefore conclude that Slug could be a target for the treatment of ATC.

Material and Methods

Cell culture

The ATC SW1736 cell line was purchased from DSMZ (Beijing, China). It was grown in RPMI1640 medium, supplemented with 10% FBS and incubated in 5% carbon dioxide and 95% air at 37°C. A monolayer of 50–70% confluent cells was used in all of the assays.

Plasmid cDNA and siRNA transfection

Slug siRNA and Slug cDNA plasmid and their controls were from our laboratory. SW1736 cells growing in 6-well plates were incubated with human Slug siRNAs (100 nmol/L). Mock-transfection was performed using a negative control siRNA (Santa Cruz Biotechnology) as control. After transfection for 48 h, the cells were washed and stored for further experiments. The knockdown efficiency of Slug was assessed using Western blot assay. To acquire the stably expressed clones, the cells were selected using G418 (400 µg/ml) for 14 days, and then routinely maintained in 200 µg/ml of G418 for further use. To determine the effect of Slug overexpression on invasion of SW1736 cells, the stably transfected Slug siRNA/SW1736 cells were transfected with Slug cDNA (50 nmol/L) for 48 h. To determine the effect of E-cadherin on invasion of SW1736 cells, the stably transfected Slug siRNA/SW1736 cells were transfected with E-cadherin siRNA (100 nmol/L) for 48 h.

Western blot analysis

Cell were trypsinized and the pellets were lysed. The protein was extracted according to the instructions. Total protein was quantified and equal amounts of protein (40 µg) was separated on 12% SDS-polyacrylamide gel electrophoresis and electrotransferred onto nitrocellulose membrane. Blots were probed with the primary antibody anti-Slug (1: 200, Santa Cruz Biotechnology, Shanghai, China) and anti-E-cadherin (1: 150, LifeSpan Biosciences, Shanghai, China). Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin antibody (1: 2000; IRDye, LI-COR, Hangzhou, China) was used as secondary antibody. β-actin was used as a housekeeping control.

Migration and Invasion assays

Migration and invasion assays were performed using a transwell membrane (8-µm pore size, 6.5-mm diameter; Guangzhou, China) in a 24-well plate according to the manufacturer's instructions. Briefly, SW1736 cells were detached from the plates, resuspended in 1% FBS, and plated in the upper insert of a 24-well chamber (1×10^5 cells/well) in serum-free medium. Chemoattractant containing 10% serum was added to the well and incubated for 24 h at 37°C. The filter inserts were

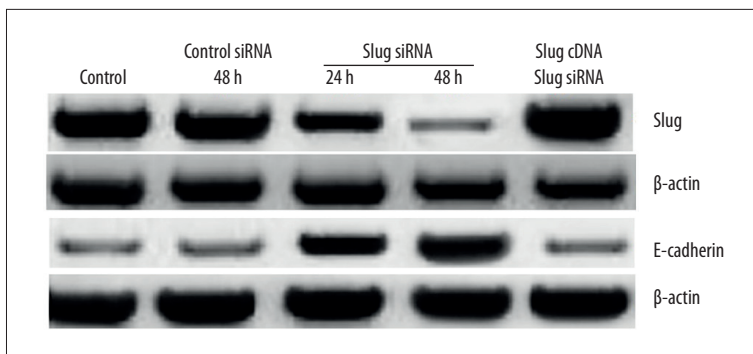


Figure 1. Effect of targeting Slug or overexpression of Slug on E-cadherin expression in SW1736 cells. SW1736 cells were transfected with 100 nM for 24 h and 48 h or the stable Slug siRNA-transfected SW1736 cells were transfected with Slug cDNA for 36 h. Slug and E-cadherin protein expression was detected by Western blot assay.

removed from the wells. Cells on the upper side of the filter were removed by scrubbing with cotton swabs. Those on the lower membrane were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature and stained with 0.1% crystal violet for 10 min. Then the invasive and migrated cells were counted in 5 random fields of each filter at 200× magnification under a microscope (IX71, OLYMPUS, JAPAN). All experiments were performed at least 3 independent times.

In vivo xenograft model of lung metastasis

Female athymic nude mice (4–5 weeks old) were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China); 30 mice (10 mice/each group) were included in the study. Briefly, Slug siRNA/SW1736 clones or control clones were injected into the right back of the mice and allowed to grow for 6 weeks. Then the mice were sacrificed. The tissues and lungs were collected. Incidence of metastatic nodes in lungs was determined by the presence of macroscopic lesions on the surface of the lung.

Immunohistochemistry

The paraffin-embedded tumor tissues were cut to 5-μm sections. Endogenous peroxidase was blocked by incubation with 3% H₂O₂ for 20 min at room temperature. Slug and E-cadherin staining was determined according to the manufacturer's instructions. Stained slides were examined under high power (×40).

Statistical analysis

Data are expressed as means ± S.E. Student's t test using SPSS18.0 software was used for the statistical analysis. The differences of statistical significance were considered to be significant when $p < 0.05$.

Results

Slug siRNA transfection inhibits Slug expression in SW1736 cells

As shown in Figure 1, Slug was richly expressed in the SW1736 cells, as shown by Western blot assay. Slug protein displayed a time-dependent reduction in the number of SW1736 cells when the SW1736 cells were transfected with Slug siRNA for 24–48 h, and reached the lowest levels at 48 h. The control siRNA did not affect Slug expression at 24–48 h.

Slug cDNA transfection reverses Slug expression in Slug siRNA/SW1736 cells

Slug siRNA stably transfected SW1736 (Slug siRNA/SW1736) clones were transiently transfected with Slug cDNA for 48 h. Slug protein expression was reversed in the Slug siRNA/SW1736 clones, as shown by Western blot assay (Figure 1). In the pre-experiment, we found that Slug expression was significantly increased at 36 h after transfection, so we selected 36 h after transfection for further studies.

Effect of targeting Slug on invasiveness of SW1736 cells

Although the Slug gene confers invasive characteristics to multiple cancer cells, its role in invasion of ATC cells is unknown. In the present study, we found that the number of cells in the lower transwell chamber were obviously decreased in the SW1736/Slug siRNA clones compared to the SW1736 or SW1736/control siRNA clones ($P < 0.01$) (Figure 2A, 2B). In contrast, the cells in the lower transwell chamber were significantly increased in the SW1736/Slug cDNA clones compared to the SW1736/Slug siRNA clones ($P < 0.01$) (Figure 2A, 2B), indicating that targeting Slug inhibits both the migration and invasiveness of SW1736 cells.

Effect of targeting Slug on E-cadherin expression

To determine the mechanism through which Slug controls the invasion of SW1736 cells, we detected the E-cadherin

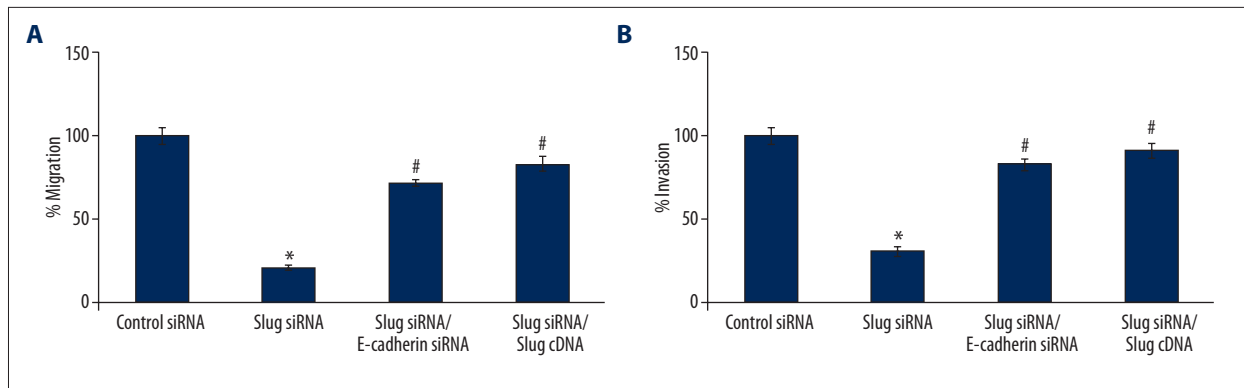


Figure 2. Effect of Slug-induced E-cadherin on migration and invasion of SW1736 cells. SW1736 cells were transfected with Slug siRNA or/and E-cadherin siRNA or Slug cDNA. Cell migration (A) and invasion (B) were detected by transwell migration and matrigel invasion assays. Vs. control, * $p < 0.01$; vs. Slug siRNA, # $P < 0.01$.

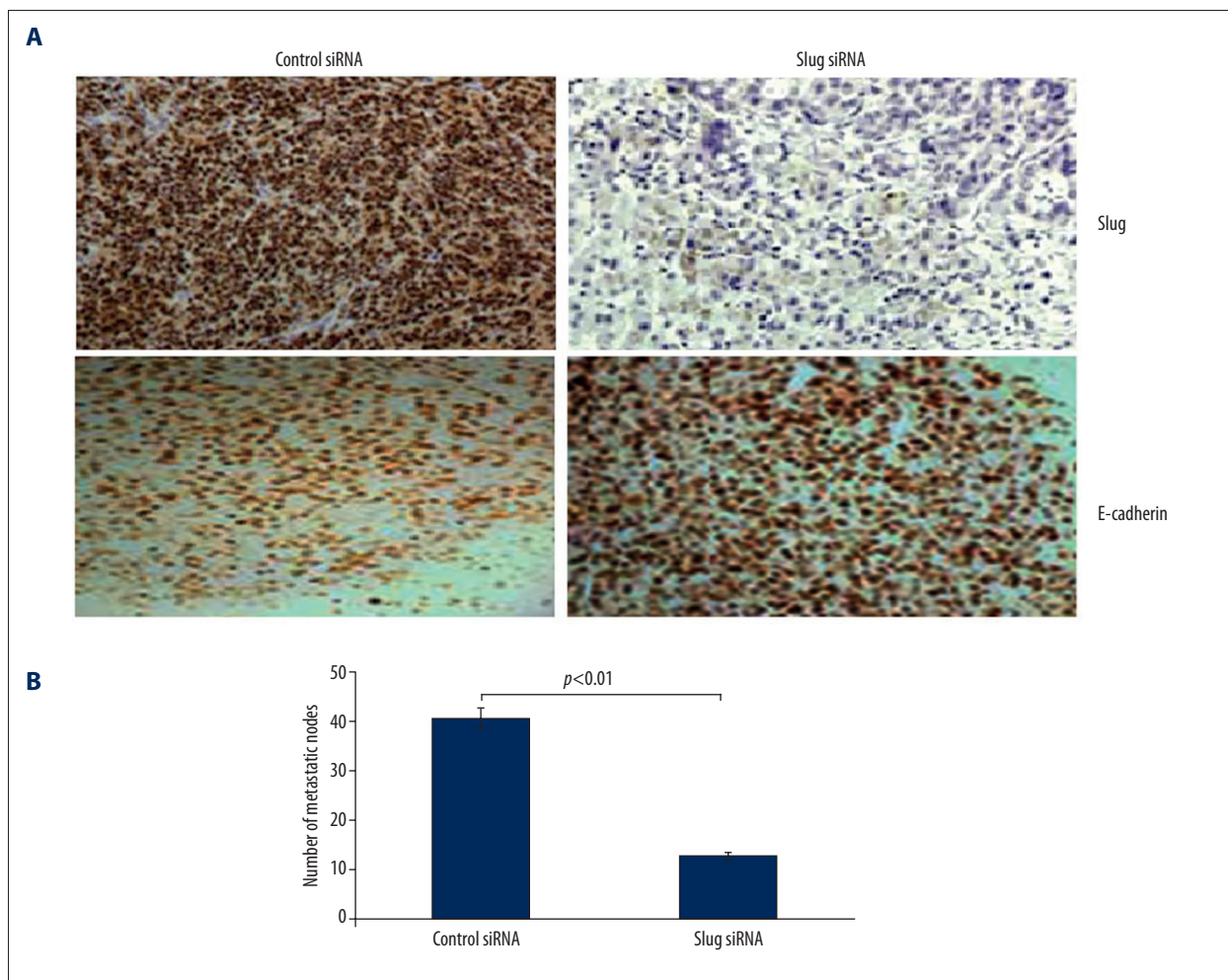


Figure 3. Targeting Slug in a xenograft model of lung metastasis. (A) Slug and E-cadherin were detected by immunohistochemistry, showing that Slug expression was significantly inhibited in Slug siRNA-transfected tumors, and E-cadherin expression was upregulated in Slug siRNA-transfected tumors. (B) The number of macroscopic metastases in Slug siRNA groups and control groups ($p < 0.01$).

expression in SW1736/Slug siRNA cells and SW1736/Slug cDNA cells by Western blot assay. The results showed that the E-cadherin expression was increased in the SW1736/Slug siRNA cells, while the E-cadherin expression was inhibited in the SW1736/Slug cDNA cells (Figure 1). These results further confirm that E-cadherin was negatively regulated by Slug in SW1736 cells.

Targeting Slug inhibits invasion of SW1736 cells by upregulation of E-cadherin

Subsequently, to detect the effect of Slug-induced E-cadherin on cell migration and invasion, the stable Slug/siRNA-transfected SW1736 cells was transfected with E-cadherin siRNA or its control siRNA for 48 h. The results showed that the E-cadherin siRNA-transfected Slug siRNA/SW1736 cells were more migratory and invasive compared to the Slug siRNA/SW1736 cells (Figure 2A, 2B).

Targeting Slug inhibits lung metastasis of SW1736 cells

SW1736, Slug siRNA/SW1736, or control siRNA/SW1736 clones were implanted in the backs of the female mice and allowed to grow for 6 weeks. Mice from each group were sacrificed, and the primary tumors were removed to determine lung metastasis. As shown in Figure 3A, Slug protein expression showed a significant decrease in the Slug siRNA/SW1736 groups compared to the control groups. However, E-cadherin expression showed a significant increase in the Slug siRNA/SW1736 groups compared to the control groups. Figure 3B shows that there were fewer macroscopic metastases nodes in the Slug siRNA/SW1736 groups than in the controls; 100% of mice injected with SW1736 cells alone developed macroscopic lung metastasis, but only 30% of the mice injected with the Slug siRNA/SW1736 clones developed similar lung metastasis.

Discussion

Slug is overexpressed in multiple cancer and is thought to promote tumor progression through regulating cell survival and invasiveness [16–19]. Several studies from our laboratory and others have shown that targeting Slug reduces cancer cells invasiveness *in vitro* and *in vivo* [11–15,17,18]. EMT has been considered the critical mechanism involved in cancer metastasis and EMT transcription factors [20]; however, the mechanism of EMT transcription factors in human cancers is largely unknown. As a strong E-cadherin repressor and major EMT

inducer, Slug plays a critical role in the invasiveness and metastasis of many of human cancers [11–19]. However, there is no previous report on Slug promoting the invasion and metastasis of ATC cells.

Here, we found that targeting Slug abrogates migration and invasion of SW1736 cell. Furthermore, restoration the Slug expression by cDNA transfection can restore the invasive ability of SW1736 cells. *In vivo*, targeting Slug also inhibits the lung metastasis of mice. It was surprising that Slug alone would play such a dominant role. However, the mechanisms by which Slug functions is not clear.

Malignant cells are characterized by loss of cellular differentiation and cell-cell adhesion, repeatedly reported to be correlated with down-regulation of E-cadherin, which was attributed to somatic mutations in some tumor types [20,21], promoter hypermethylation [22], or the action of Slug and Snail transcriptional repressor [23,24]. Loss of E-cadherin is associated with the development of metastatic dissemination, invasive carcinoma, and poor clinical prognosis [25,26]. Catalano et al. reported that inducing E-cadherin expression and the proper membrane localization of the E-cadherin/ β -catenin complex by histone deacetylase reduced cancer cell migration and invasion [27]. Therefore, loss of E-cadherin expression may contribute to tumorigenesis.

To determine whether Slug promotes invasion by repressing the E-cadherin expression, we detected the E-cadherin expression in Slug siRNA or Slug cDNA-transfected SW1736 cells. The results showed that siRNA-mediated down-regulation of Slug expression increased E-cadherin expression in SW1736 cells, as demonstrated by Western blot analysis. However, after the Slug/siRNA-transfected SW1736 cells were transfected with Slug cDNA or E-cadherin siRNA, the E-cadherin expression was decreased, and cell migratory and invasive ability was restored. Studies *in vivo* have also demonstrated that tumor metastasis can be achieved by Slug inhibition and E-cadherin upregulation. These data demonstrate that Slug promotes invasion of SW1736 cells by repressing E-cadherin expression.

Conclusions

Targeting Slug could potentially activate E-cadherin, resulting in the inhibition of cell invasiveness *in vitro* and metastasis of ATC SW1736 cells *in vivo*. Slug is an effective target for the treatment of ATC.

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