Original Article
Targeting of slug sensitizes anaplastic thyroid carcinoma SW1736 cells to doxorubicin via PUMA upregulation

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Abstract: Objective: Anaplastic thyroid carcinoma (ATC) is one of the most aggressive human cancers and often shows resistance to multimodal therapeutic approaches. It has been shown that the transcriptional repressor Slug inhibits the chemotherapeutic agent-induced apoptosis of cancer cells. We evaluated whether targeting of Slug could augment doxorubicin (DOX)-induced apoptosis of ATC cells. We also determined changes in PUMA (p53-upregulated modulator of apoptosis) expression levels to identify possible mechanisms of their combined actions.

Methods SW1736 cells were transfected with Slug siRNA or/and PUMA siRNA and then exposed to DOX (0.1, 1, and 5 mM) for selected times. Scrambled siRNA was used as a control. The effects on cell viability were determined via MTT assay. Apoptosis was assessed using TUNEL assays and annexin V staining, and was confirmed by flow cytometry analyses. Slug and PUMA levels were determined using western blotting and immunofluorescence analyses. We used a subcutaneous implanted tumor model of SW1736 cells in nude mice to assess the effects of Slug silencing in combination with DOX on tumor development. Apoptosis was assessed via TUNEL assay. Results Targeting of Slug using siRNA combined with DOX led to lower cell viability than treatment with DOX alone in SW1736 cells. TUNEL and flow cytometry analyses showed that targeting of Slug enhanced DOX-induced apoptosis of SW1736 cells. In addition, targeting of Slug increased PUMA expression, and targeting of PUMA restored the chemoresistance of SW1736/Slug siRNA cells to DOX. Conclusions Knockdown of Slug enhanced the antitumor activity of DOX in SW1736 cells via induction of PUMA upregulation. Our results suggest that targeting of Slug has good potential for the development of new therapeutic strategies for ATC.

Keywords: Anaplastic thyroid carcinoma, chemotherapy, slug, PUMA

Introduction

Anaplastic thyroid cancer (ATC) ranks among the most lethal of all human malignancies [1]. ATC usually presents between the 6th and 7th decades of life as a rapidly enlarging neck mass that extends locally and disseminates to regional nodes and distant sites. Multimodal therapy, including surgery, chemotherapy, and radiotherapy, has only limited benefits in locoregional control of the disease [2]. Because of the overall poor prognosis and extremely short survival times, close early monitoring of response to treatment is warranted.

There is currently no effective therapy for ATC. Traditional cytotoxic chemotherapies, such as doxorubicin (DOX) and paclitaxel, are highly toxic and largely ineffective at prolonging survival in ATC patients [3]. Results from a phase II trial with paclitaxel showed a short-term total response rate of 53%, but no change in disease outcomes [4]. Radiation therapy, alone or in conjunction with DOX, did not improve overall survival [3]. The poor outcomes for chemotherapy are in part the result of elevated levels and activities of multidrug resistant proteins [5], strong activation of prosurvival pathways, and a high degree of chromosomal instability and aneuploidy [6]. It is important to understand the mechanisms underlying this resistance so that a treatment can be developed to sensitize ATC to chemotherapy.

It has been found that Slug, a member of the Snail family of zinc-finger transcription factors,
proteins. The cultures were free of Mycoplasma species. In all of the assays, a monolayer of cells with 50-70% confluence was used. All protocols followed the manufacturer’s instructions.

Reagents and plasmid

DOX was purchased from Sigma-Aldrich (Hangzhou, China). Anti-Slug, anti-PUMA, and anti-β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Slug siRNA, PUMA siRNA, and the control siRNA were purchased from Santa Cruz Biotechnology.

siRNA/shRNA transfection

SW1736 cells were transfected with Slug siRNA/shRNA and control siRNA/shRNA for 24-72 h [shRNA for 24 h] using Lipofectamine 2000 according to the manufacturer’s instructions. For stable Slug siRNA/shRNA transfection 24 h after Slug siRNA/shRNA or control siRNA/shRNA transfection, the cells were divided into 96-well plates and subjected to G418 (1 mg/mL) selection for 2 weeks. Transcriptional silencing of Slug was assessed using a western blot assay. To determine the role of PUMA expression, SW1736 cells stably transfected with Slug siRNA were transiently transfected with PUMA siRNA or control siRNA for 24-48 h. All transfection experiments were performed at least three times.

MTT assay

SW1736 cells were plated in triplicate in 96-well microtiter plates (1 × 10⁴ cells/well) and exposed to DOX (0.1, 1, and 5 mM) for 24, 48, and 72 h. Cell viability was determined by MTT assay. For each DOX concentration and incubation time, 20 µL of 5 mg/mL MTT was added to the well and incubated for 4 h at 37°C. Formazan crystals were dissolved in 100 µL of anhydrous isopropanol with 0.1 N HCl (Sigma-Aldrich, St. Louis, MO, USA). The optical density was determined using a Bio-Rad microculture plate reader (Bio-Rad, Hercules, CA, USA) at 570 nm. Each assay was performed in triplicate. Absorbance values were normalized to the value for vehicle-treated cells to determine percentage survival.

TUNEL assay

SW1736 cells were plated in triplicate and exposed to DOX (0.1, 1, and 5 µm) for 24, 48,
and 72 h. Cells were then plated on polylysine-coated slides, fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) for 1 h at 25°C, rinsed with 0.1 M PBS, pH 7.4, and permeabilized with 1% Triton X-100 in 0.01 M citrate buffer, pH 6.0. DNA fragmentation was detected using a TUNEL detection kit (Roche Clinical Laboratories, Indianapolis, IN, USA) that specifically labels the 3′-hydroxyl terminus of DNA strand breaks with fluorescein isothiocyanate (FITC)-conjugated dUTP. DNA was also labeled with FITC DNA-binding dye for 5 min. FITC labeling was observed under a fluorescence microscope. The percentage of apoptotic cells was calculated as the number of apoptotic cells per number of total cells × 100%. Slides were deparaffinized, hydrated, washed, permeabilized, and incubated with the TUNEL reaction mixture for the apoptosis assay. After washing, slides were observed under a fluorescence microscope (Nikon, Melville, NY, USA).

**Flow cytometry**

Phosphatidylserine redistribution in the plasma membrane was measured using an annexin V-FITC/PI apoptosis detection kit (Abcam, Hangzhou, Zhejiang, China). After 24-72 h of treatment, 1 × 10^5 harvested cells were suspended in 500 mL of annexin V binding buffer. Annexin V-FITC (0.5 mL) and 5 mL of PI were added and incubated for 15 min in the dark. Binding buffer (400 mL) was added to each sample. The stained cells were analyzed directly by flow cytometry using the Cell Quest program (Becton Dickinson, San Jose, CA, USA).

**Western blotting**

After treatment, whole-cell extracts were prepared using lysis buffer containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 10 mM KCl, 1 mM EDTA (pH 8.0), 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 mM Na_3VO_4, 5 mM NaF, 20% glycerol, and 1% (v/v) mammalian protease inhibitor (Sigma-Aldrich), and the samples were heated at 95°C for 15 min.

Protein was quantified using the Bradford assay (Bio-Rad), and equal amounts of protein were separated on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, USA). Blots were probed with the indicated antibodies and developed using a Pierce chemiluminescence substrate (Thermo Fisher Scientific, Rockford, IL, USA).

**Immunofluorescence**

SW1736 cells at the end of treatment were plated in six-well chamber slides for 24 h. The cells were then fixed in 4% paraformaldehyde for 10 min at room temperature, permeabilized with 0.1% Triton X-100 in 0.01 M PBS (pH 7.4) containing 0.2% bovine serum albumin, air dried, and rehydrated in PBS. Then cells were incubated with anti-Slug or anti-PUMA antibody diluted 1:150 in PBS containing 1% normal goat serum for 2 h at room temperature. Negative controls were prepared by omitting the primary antibody. After three washes with PBS for 10 min, an anti-rabbit IgG FITC-conjugated secondary antibody (Santa Cruz Biotechnology) diluted 1:250 in PBS was added for 2 h at room temperature. Cells were then washed in PBS and stained with 10 mg/mL Hoechst 33258 (Sigma-Aldrich) for 10 min at room temperature to counterstain the DNA. Cells were observed using a Zeiss Axiohot fluorescence microscope (Axio-Cam MRc; Zeiss, Oberkochen, Germany). Images were acquired using a digital video camera and Axiovision Zeiss software (Zeiss).

**In vivo studies**

Animal studies were approved by the Animal Care and Use Committee and conducted in accordance with NIH guidelines. SW1736, Slug shRNA/SW1736, or control shRNA/SW1736 cells were suspended in Matrigel (5 × 10^6 cells/200 mL) and inoculated subcutaneously into the right flank of 4- to 6-week-old female athymic nude (nu/nu) mice. Three weeks after injection, when the tumor size reached approximately 20-35 mm^2, the tumor-bearing mice were intravenously injected with DOX (1.2 mg/kg) once per day for 3 days. The tumor size in treated mice was measured after treatment. The mice were sacrificed 21 days after the first DOX injection, and tumors were dissected for immunohistochemistry and TUNEL staining.

**Statistical analysis**

All statistical analyses were performed using SPSS version 22 for Windows (Chicago, IL, USA). Data are reported as the mean ± SD.
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Analysis of variance (ANOVA) was used when more than two groups were involved, and Student’s t-test was further used to analyze differences between groups. *P < 0.05 was considered statistically significant.

Results

Slug siRNA transfection inhibits Slug expression

Figure 1A shows that SW1736 cells had a high background level of Slug protein expression. When SW1736 cells were transfected with Slug siRNA for 24-72 h, there was a time-dependent decrease in Slug protein expression, with the lowest expression observed at 72 h. Scrambled siRNA transfection for 72 h did not affect Slug protein expression in SW1736 cells (Figure 1A).

An indirect immunofluorescence assay confirmed that Slug protein expression decreased in a time-dependent manner with Slug siRNA transfection (Figure 1C).

Slug knockdown induces PUMA expression

We next determined whether Slug knockdown upregulates PUMA expression. SW1736 cells expressed a low background level of PUMA protein, as assessed by western blotting (Figure 1B) and immunofluorescence (Figure 1D). However, knockdown of Slug expression via siRNA transfection significantly increased PUMA protein expression, which the highest expression observed at 24 h (Figure 1B, 1D). Western blotting revealed that scrambled siRNA transfection for 72 h did not affect PUMA protein expression in SW1736 cells (Figure 1B). Immuno-
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fluorescence confirmed these results (data not shown).

**Slug knockdown induces apoptosis and inhibits cell viability**

SW1736 cells were transfected with Slug siRNA or control siRNA for 72 h. Cell viability was assessed by MTT assay. The results show that Slug siRNA transfection significantly inhibited cell growth (>35%) compared to control siRNA transfection (P < 0.05; Figure 2A).

Translocation of phosphatidylserine to the outer surface of the cytoplasmic membrane is an early event in apoptosis. Annexin V and PI binding were used to evaluate the surface expression of phosphatidylserine. Staining with annexin V alone indicates early apoptotic changes and intact cell membranes, whereas staining with both annexin V and PI indicates membrane disintegration consistent with necrosis or a late stage of apoptosis. As shown in Figure 2B, SW1736 cells transfected with Slug siRNA exhibited a significant increase in apoptosis and necrosis compared to cells transfected with control siRNA (P < 0.05). This is confirmed by TUNEL results showing an increase in cell apoptosis after Slug siRNA transfection (Figure 2C).

**Slug knockdown sensitizes SW1736 cells to DOX**

SW1736 cells were treated with DOX (0.1, 1, and 5 mM) for 72 h. DOX did not significantly inhibit growth, and induced apoptosis as assessed by MTT assay (Figure 2A). Flow cytometry (Figure 2B) and a TUNEL assay (Figure 2C) suggested that SW1736 cells were resistant to DOX treatment.

We further examined the involvement of Slug silencing during DOX-induced apoptosis and growth inhibition. SW1736 cells transfected with Slug siRNA were exposed to DOX (0.1, 1, and 5 mM) for 72 h. The results show that combined Slug siRNA and DOX treatment enhanced apoptosis and inhibited cell growth (Figure 2A-C). The inhibitory effect of combined Slug siRNA and DOX was greater than that of Slug siRNA or DOX alone (Figure 2A-C).
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Slug knockdown enhances DOX-induced apoptosis via PUMA-dependent signaling

The mechanisms by which Slug enhances the chemoresistance of SW1736 cells to DOX remain unknown. It has been shown that Slug attenuation sensitizes cells to apoptosis and that Slug represses PUMA. To verify whether Slug knockdown enhanced DOX-induced apoptosis of SW1736 cells via activation of PUMA, SW1736 cells stably transfected with Slug siRNA were transfected with PUMA siRNA for 24 h and then treated with DOX (0.1, 1, and 5 mM) for 72 h. Transfection with PUMA siRNA blocked Slug-siRNA-induced PUMA expression (data not shown) and rescued the chemoresistance of SW1736 cells to DOX, as shown by MTT assay (Figure 2A), flow cytometry (Figure 2B), and TUNEL assay (Figure 2C).

DOX has a significant therapeutic effect on ATC tumors treated with Slug siRNA

To elucidate the synergistic effects of DOX and Slug siRNA, inhibitory effects on tumor growth were examined in vivo. Combined DOX and Slug siRNA significantly inhibited tumor growth ($P < 0.01$) compared to DOX or Slug siRNA alone at day 21 (Figure 3A). DOX alone had a slight therapeutic effect compared to controls, and Slug siRNA significantly inhibited tumor growth (Figure 3A). To examine the therapeutic effects of siRNA and DOX on SW1736 tumor cells, TUNEL staining was performed to reveal apoptotic cells. Approximately 11.4% of SW1736 cells treated with Slug siRNA and DOX underwent apoptosis, in comparison to 6.1% of SW1736 cells treated with Slug siRNA alone. These values are significantly greater than for cells treated with DOX alone ($P < 0.01$ and $P < 0.05$, respectively; Figure 3B).

Discussion

ATC is characterized by extremely fast growth and undifferentiated features, which pose a major challenge for current treatment modalities. Despite multimodal approaches, the prognosis for ATC is poor, and median overall survival has not improved over the last 50 years [13]. Because ATC is refractory to conventional chemotherapy, radiotherapy, and radioiodine ($^{131}$I) [14], new therapeutic approaches are urgently needed.

Slug is a zinc-finger transcription factor that is critical for embryonic development [15] and is overexpressed in many cancers, including ATC [12, 16]. However, the role of Slug in ATC is unknown. In the present study, we showed that Slug is overexpressed in ATC SW1736 cells. Slug knockdown had a remarkable inhibitory effect on the growth of SW1736 cells in vitro and in vivo, suggesting that targeting of Slug could be an effective treatment for ATC.

Previous studies have revealed that Slug overexpression has a radioprotective function in TK6 cells, suggesting that Slug could be used in a gene therapy approach for radioprotection of
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normal tissues [17]. Mancini et al. reported that Slug overexpression contributed to apoptosis resistance, prolonged survival, and increased the imatinib resistance of chronic myelogenous leukemia progenitor cells [18].

Treatment options for ATC include surgery, chemotherapy, and radiotherapy, but all these treatments, especially if used alone, generally fail to control local disease. ATC cannot be regarded as a very chemo-sensitive tumor. DOX and its analogs are typically used for treatment, but the response rate is typically not more than 20% [19]. In the present study, we found that treatment with up to 5 mM DOX for 72 h resulted in minimal cell apoptosis, suggesting that SW1736 cells are resistant to DOX treatment. However, Slug downregulation significantly increased DOX-induced apoptosis and growth inhibition in vitro and in vivo. These results suggest that Slug overexpression increases chemoresistance, and vice versa.

Although Slug is inherently resistant to DOX-induced apoptosis, the underlying molecular mechanism is unknown. It has recently been reported that Slug antagonizes apoptosis of hematopoietic progenitor cells by repressing PUMA transactivation [7]. Furthermore, Slug downregulation facilitates apoptosis of fibroblast-like synoviocytes by increasing PUMA transactivation [20]. Our cell culture studies showed that Slug knockdown increased PUMA expression and sensitized SW1736 cells to DOX. In addition, knockdown of PUMA expression rescued the chemoresistance of SW1736 cells to DOX, suggesting that Slug silencing sensitizes SW1736 cells to DOX via a PUMA-dependent pathway. Our in vivo studies using DOX in mice with SW1736 xenografts confirmed that Slug knockdown combined with DOX administration significantly inhibited tumor growth and induced apoptosis.

Our results also showed that Slug knockdown sensitized SW1736 cells to DOX-induced apoptosis via PUMA upregulation. The involvement of PUMA in apoptosis is supported by the observation that PUMA siRNA lowered the threshold for induction of apoptosis by DOX.

Conclusions

Our study emphasizes an important function of Slug in the chemoresistance of SW1736 cells to DOX. Slug knockdown induces tumor cell apoptosis and inhibits cell growth, and sensitizes SW1736 cells to DOX. All these effects were achieved via PUMA upregulation. Slug knockdown therefore has potential for the development of new therapeutic strategies to improve chemotherapy for ATC.

Disclosure of conflict of interest

None.

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References

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