

Knockdown of S100A4 chemosensitizes human laryngeal carcinoma cells *in vitro* through inhibition of Slug

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Abstract. – **OBJECTIVE:** S100A4 and Slug are known to be closely involved in resistance to chemotherapy. Furthermore, Slug signal was regulated by S100A4. Targeted therapy reducing S100A4 expression and Slug pathway activity may overcome the chemoresistance of human cancers. We hypothesized that over-expression of S100A4 and Slug was associated with the resistance to cisplatin of laryngeal carcinoma Hep-2 cells. We explored whether S100A4 silencing inhibited Slug, resulting in sensitization of laryngeal carcinoma Hep-2 cells to cisplatin.

MATERIALS AND METHODS: We investigated the effects of S100A4 and Slug silencing by siRNA transfection on chemosensitivity to cisplatin (DDP) in Hep-2 cells *in vitro*. In order to confirm the correlation between S100A4 and Slug signals, siRNA transfected Hep-2 cells were over-expressed by pSlug transfection, then explored the effect of S100A4 silencing on chemosensitivity to cisplatin (DDP) in Hep-2 cells *in vitro*. Real-time RT-PCR and Western blotting confirmed the presence of S100A4 mRNA, Slug mRNA and proteins in Hep-2 cells.

RESULTS: We found that resistance or insensitivity of Hep-2 cells to cisplatin might be associated with S100A4 and Slug expression. Knockdown of S100A4 and Slug markedly enhanced the cisplatin-induced suppression of Hep-2 cell growth and increased apoptosis. Knockdown of S100A4 may significantly reduce the levels of S100A4 mRNA, Slug mRNA and proteins, in cisplatin-treated Hep-2 cells. Re-expression of Slug in S100A4 siRNA transfected Hep-2 cells restored the cisplatin resistance in the Hep-2 cells.

CONCLUSIONS: Overexpression of S100A4 may be associated with the resistance to cisplatin of laryngeal carcinoma Hep-2 cells. Knockdown of S100A4 enhances the sensitivity to cisplatin of laryngeal carcinoma cells via inhibition of Slug expression.

Key Words:

Laryngeal carcinoma, Chemotherapy, Cisplatin, S100A4, Slug.

Introduction

Laryngeal carcinoma is one of the most common head and neck cancers. Current therapeutic strategies for the early stages of laryngeal carcinoma include various types of larynx-conserving surgery, or radiotherapy. For advanced laryngeal carcinoma, combined therapies are often used, including concurrent chemo-radiotherapy or total laryngectomy, with possible adjuvant therapy¹. However, the survival rate has not improved over the last few decades² because of resistance to chemo-radiotherapy and late metastasis^{3,4}. Thus, overcoming the resistance to chemo-radiotherapy of laryngeal carcinoma is a challenge in cancer therapy.

S100A4 (also known as mts1), a calcium-binding protein was associated with invasion and metastasis of various cancer types cancer cells, such as thyroid, renal, hepatocellular, Wilms tumor, osteosarcoma and laryngeal cancer⁵⁻¹⁰. Patrick et al¹¹ has found knockdown of S100A4 resulted in an increase in the sensitivity of pancreatic ductal adenocarcinoma cell lines to gemcitabine as well as an increase in the number of apoptotic cells. They also found that the combined effect of S100A4 knockdown and treatment with gemcitabine resulted in a clear augmentation in the activation of the apoptotic mediator proteins compared with control cells. Therefore, S100A4 activity is closely associated with resistance to chemotherapy and, when this pathway is inhibited, the sensitivity to chemotherapy is enhanced.

Slug, a member of the Snail family of transcription factors, plays a crucial role in the regulation of epithelial-mesenchymal transition (EMT) by suppressing several epithelial markers and adhesion molecules including E-cadherin. It is detectable in many types of cancer, and its presence has been associated with poor prognosis in many malignant tumors¹²⁻¹⁵. Previous studies have demonstrated that Slug overexpression exhibits a

radioprotective function in TK6 cells demonstrating that it has potential as a candidate for gene therapeutic radioprotection of normal tissues¹⁶. Mancini et al¹⁷ has demonstrated that Slug overexpression contributes to apoptosis resistance in leukemic progenitors. She reported that Slug overexpression was involved in prolonged survival and imatinib (IM) resistance of chronic myelogenous leukemia (CML) progenitors. Slug is also an important modulator of the therapeutic response of cancer cells and is potentially useful as a sensitizer in cancer therapy^{18,19}.

Abnormal expression of S100A4 is associated with the actions of multiple signal transduction pathways, including the Slug signaling pathway²⁰. However, no report has yet explored the possible correlation between S100A4 expression and resistance to chemotherapy of laryngeal carcinoma. And whether the correlation was through Slug signal is not clear.

In this study, we hypothesized that overexpression of S100A4 and Slug was associated with resistance to cisplatin of laryngeal carcinoma Hep-2 cells. We explored whether the effect of S100A4 and Slug silencing sensitized laryngeal carcinoma Hep-2 cells to cisplatin. Next, we explored whether the effect of S100A4/Slug signal regulate chemosensitivity to cisplatin in laryngeal carcinoma Hep-2 cells.

Materials and Methods

Cell Lines

The human Hep-2 cell lines was obtained from American Type Culture Collection (Rockville, MD, USA). The cell lines were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal calf serum (FCS), 1% penicillin and streptomycin at 37°C in 5% CO₂.

S100A4 siRNA and Slug siRNA Clones

siRNAs (S100A4 siRNA or Slug siRNA or their control siRNA) were dissolved in buffer [100 mmol/L potassium acetate, 30 nmol/L HEPES-KOH, 2 nmol/L magnesium acetate (pH 7.4)] to a final concentration of 20 μ mol/L, heated to 90°C for 60 s and incubated at 37°C for 60 min before use to disrupt any higher order aggregates formed during synthesis. Hep-2 cells were plated into 35-mm 6-well trays and allowed to adhere for 24 hours. In all, 8 μ L siPORT Amine transfection reagent (Ambion, Inc., Austin, TX, USA) per well were added to serum-free medium for a final complexing volume of 200 μ L, vortexed,

and incubated at room temperature for 15 minutes. The transfection reagent/siRNA complexes (S100A4 siRNA or Slug siRNA or their control siRNA) were added to the wells containing 800 μ L medium with 10% fetal bovine serum and incubated in normal cell culture conditions for 6 hs, after which 1 mL Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) was added. Assays were done 48 hs post-transfection.

S100A4 siRNA/Slug Transient clones

The full coding region of human Slug (pSlug-EGFP or pEGFP expression plasmid) was from Dr. Zhang K (General Surgery, the Affiliated Hospital of Medical College, QingDao University, China), of which the DNA sequence was confirmed. pSlug or pEGFP expression plasmid was transfected into the S100A4 siRNA/ Hep-2 cells for 48 hs as the method above.

Cytotoxicity Assay

Cisplatin (5 mg) was dissolved in 1,000- μ L dimethyl sulfoxide (DMSO) and used at 5 μ g/ μ L in the experiments. Cytotoxicity was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT, Trevigen, Inc., Gaithersburg, MD, USA) in accordance with the manufacturer's instructions. Briefly, logarithmically growing cells were plated at 5×10^3 cells (Hep-2, Hep-2/siRNA (S100A4 or Slug), Hep-2/siRNA(S100A4)/pSlug) per well in 96-well plates, allowed to adhere overnight, and were cultured in the presence or absence of 5 μ g/ μ L cisplatin. Cisplatin induced cytotoxicity was determined after 24 hours of exposure. Plates were read using a Vmax microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 570 nm corrected to 650 nm and normalized to controls. Each independent experiment was done thrice, with 10 determinations for each condition tested. At identical time points, cells were trypsinized to form a single cell suspension. Intact cells, determined by trypan blue exclusion, were counted using a Neubauer hemocytometer (Hausser Scientific, Horsham, PA, USA). Cell counts were used to confirm MTT results.

Flow Cytometry

Cells (1×10^6) were harvested and the pellets were washed twice with phosphate buffered saline (PBS). Cells were then fixed in cold 70% ethanol added dropwise while vortexing gently. Fixed cells were kept overnight at 4°C. Cells were centrifuged and pellets were resuspended in 1 mL

propidium iodide/RNase staining buffer (BD Biosciences, San Jose, CA, USA). Reactions were incubated for 20 min at 4°C and protected from the light. Samples were analyzed by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences). For each sample, at least 2×10^4 cells were analyzed. Cell cycle distribution was calculated by CellQuest software (BD Biosciences).

Western Blotting

The whole-cell lysates (40 μ g) were fractionated by 10% gradient SDS-PAGE. After SDS-PAGE, the proteins were electrotransferred onto nitrocellulose membranes and probed with appropriate primary (S100A4 and Slug) and secondary antibodies. Antigen-antibody reaction was detected with Western Lightning chemiluminescence reagent (Perkin-Elmer, Waltham, MA, USA). The bands obtained were quantified using AlphaEaseFC (FluorChem 8900) software from Alpha Innotech Corp. (San Leandro, CA, USA).

Quantitative Reverse Transcription-PCR

Real-time amplification of S100A4 and Slug was done from 2 μ L cDNA prepared from 2 μ g of total RNA. The following primers were used: S100A4, forward 5'-ATTTCTGCCAGGACCGCTTCTACT-3' and reverse 5'-CAGTTTGTATCCG-GCAAAGTGGCT-3'; Slug forward 5'-TCACAC-CAAGGAAGAGAATGGCCT-3' and reverse 5'-

AATGACAACCAGCAAGAAAGCGGG-3'; and β -actin, forward 5'-ATCTGGCACCACACCTTC-TACAATGAGCTGCG-3' and reverse 5'-CGT-CATACTCCTGCTTGCTGATCCACATCTGC-3'. PCR reaction standardization kits were obtained from Epicentre. The cDNA was amplified with an initial denaturation at 95°C for 10 s followed by sequential cycles of denaturation at 94°C for 45 s, annealing at 55°C for 10 s, and extension at 72°C for 1 min for 30 cycles, with a final extension at 72°C for 7 min.

Statistical Analysis

All experiments were conducted in triplicate and carried out on three or more separate occasions. Data presented are means of the three or more independent experiments $\pm$ SE. Statistically significant differences were determined by two-tailed unpaired Student's *t* test and were defined as $*p < 0.05$.

Results

Cisplatin Treatment Did Not Affect S100A4 and Slug Expression

Hep-2 cells were treated with 0–5 μ g/ μ L cisplatin for 24 h. Western blot and quantitative reverse transcription-PCR was used to detect S100A4 mRNA, Slug mRNA and protein. As shown in Figure 1A and B, cisplatin treatment

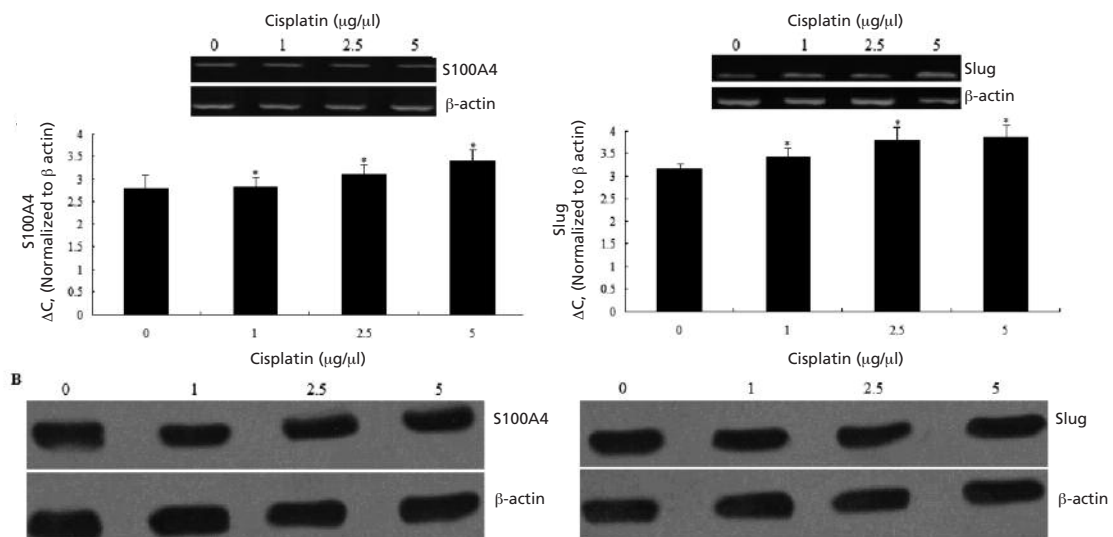


Figure 1. Cisplatin treatment does not influence S100A4 and Slug levels in Hep-2 cells *in vitro*. **A**, Hep-2 cells line was treated with indicated concentrations of cisplatin for 24 h *in vitro* and the S100A4 mRNA and Slug mRNA levels was analyzed by quantitative reverse transcription-PCR. **B**, Hep-2 cells line was treated with indicated concentrations of cisplatin for 24 h *in vitro* and the S100A4 and Slug protein levels was analyzed by western blot. All measurements are shown as a percentage of basal and are means $\pm$ SE for three independent experiments. Vs control, $*p > 0.05$.

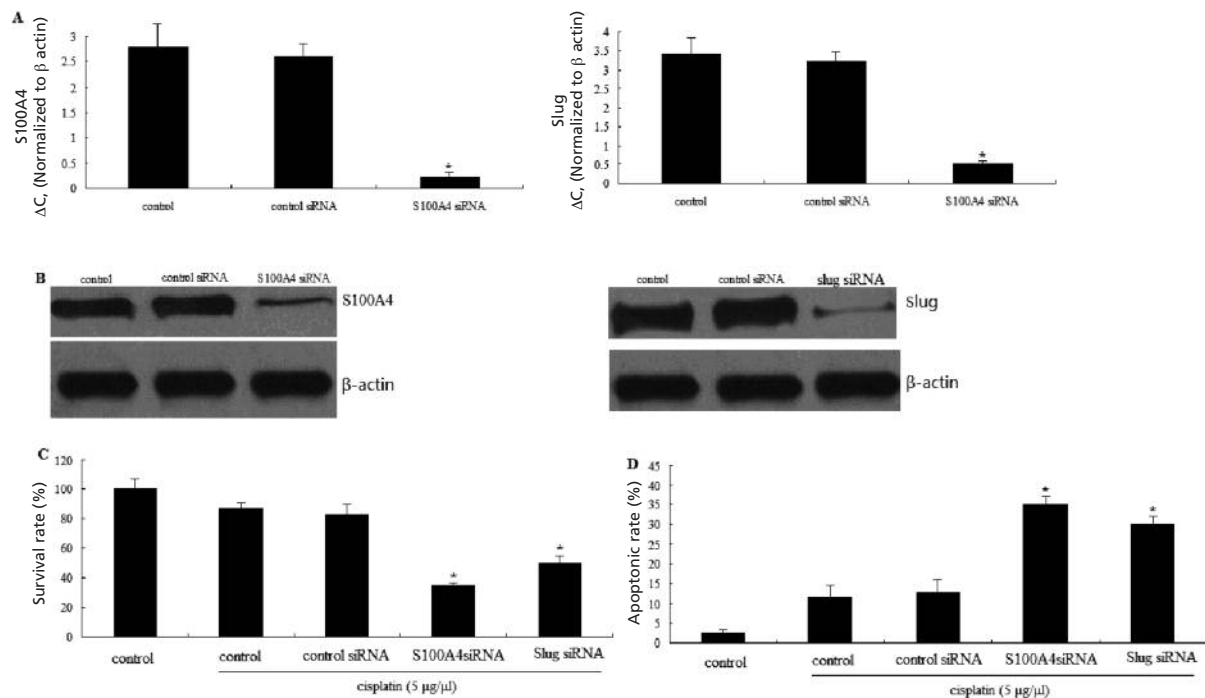


Figure 2. Effect of siRNA-mediated knockdown of S100A4 and Slug expression on cell viability and apoptosis in Hep-2 cells. **A**, Hep-2 cells were transfected with either Slug siRNA or S100A4 siRNA or control siRNA and 48 h, quantitative reverse transcription-PCR was used to detect S100A4 mRNA and Slug mRNA level. **B**, Hep-2 cells were transfected with either Slug siRNA or S100A4 siRNA or control siRNA and 48 h, western blot was used to detect S100A4 and Slug protein level. **C**, Hep-2 cells were transfected with either Slug siRNA or S100A4 siRNA or control siRNA and 48 h, then the cells were subjected to cisplatin (5 $\mu\text{g}/\mu\text{l}$), the number of viable cells was quantified using the MTT assay over a period of 3d. **D**, Hep-2 cells were transfected with either Slug siRNA or S100A4 siRNA or control siRNA and 48 h, then the cells were subjected to cisplatin (5 $\mu\text{g}/\mu\text{l}$), the number of apoptotic cells was quantified using the flow cytometry assay over a period of 3d. Vs Control, * $p < 0.05$.

for 24 hours had no significant effect on S100A4 mRNA, Slug mRNA and protein expression.

S100A4 and Slug Knockdown Promotes Cisplatin-Induced Cytotoxicity

Hep-2 cells were chosen to study the effects of S100A4 or Slug knockdown, as the Hep-2 cell inherently express high level of S100A4 or Slug (Figure 1). S100A4 and Slug expression was suppressed by transfection of S100A4-specific siRNA and Slug-specific siRNA. 48 hs following transfection of siRNA, cells were subjected to cisplatin (5 $\mu\text{g}/\mu\text{l}$) cytotoxicity assay as described previously. Continued suppression of S100A4 and Slug expression of up to 95% and 85% was confirmed by quantitative reverse transcription-PCR, 48 hs following siRNA transfection, respectively (Figure 2A). Continued suppression of S100A4 and Slug expression was confirmed by Western blotting, 48 hours following siRNA transfection, respectively (Figure 2B). S100A4 or Slug knockdown combined with cisplatin (5 $\mu\text{g}/\mu\text{l}$) resulted in a 70% or 55% decrease in the survival rate, as determined

by MTT assay, relative to control siRNA transfectants (Figure 2C). Apoptotic cells induced by exposure to cisplatin (5 $\mu\text{g}/\mu\text{l}$) resulted in a 40% or 35% increase following transfection of S100A4 (Slug)-specific siRNA but not control siRNA (Figure 2D). Knockdown of S100A4 or Slug alone did not affect survival and apoptosis of Hep-2 cells (data not shown).

Knockdown of S100A4 Inhibits Slug Expression

Hep-2 cells were transfected with S100A4-specific siRNA for 48 hs, Slug expression was significantly suppressed by Western blotting (Figure 3B) and quantitative reverse transcription-PCR assay (Figure 3A).

Knockdown of S100A4 Promotes Cisplatin-induced Cytotoxicity by Suppressing Slug

S100A4 knockdown resulted in significant decrease in the survival rate (Figure 2C), and sig-

nificant increase in the apoptotic cells (Figure 2D) induced by exposure to cisplatin (5 $\mu\text{g}/\mu\text{l}$). However, when the S100A4 siRNA transfected Hep-2 cells were transfected with the full coding region of human Slug (pSlug) to restore the Slug level (Figure 3B), the increase in apoptotic cells induced by S100A4 knockdown was significantly reduced (Figure 3C), and the survival rate was significantly increased (Figure 3D). Transfection

of pSlug cDNA alone did not affect survival and apoptosis of Hep-2 cells (data not shown).

Discussion

Previous study showed a significant correlation between Slug expression levels and cisplatin resistance in laryngeal carcinoma Hep-2 cell line²¹ and showed that Slug expression is widely up-regulated in laryngeal carcinoma specimens²². We postulated that the identification of upstream factors that are responsible for the promotion of Slug expression in laryngeal carcinoma may further illuminate our understanding of the molecular mechanisms underlying chemoresistance in laryngeal carcinoma.

S100A4 is a well established marker and mediator of metastatic disease and tumor growth, but the exact mechanisms responsible for the effects are less well defined. It has found both extracellular and intracellular S100A4 participates in the regulation of cell death. Prosurvival functions have been described both in malignant²³ and nonmalignant cell systems²⁴. In osteosarcoma, positive S100A4 expression more frequently occurred in tissues with advanced clinical stage, positive distant metastasis and poor response to chemotherapy²⁵. In colon cancer human cells, S100A4 overexpression decreases the sensitivity of HT29 cells to MTX, whereas its knockdown causes chemosensitization toward MTX²⁶. In squamous cell laryngeal cancer, S100A4 promoted Hep-2 cell invasion via NF- κ B/MMP-9 signal pathway²⁷.

It has reported S100A4 is positively associated with slug signaling pathway²⁰. We confirmed the positive correlation between S100A4 and Slug expression in Hep-2 cell line and, using Hep-2 cell line expressing an S100A4 siRNA, we showed that knockdown of S100A4 resulted in a decreased expression of the Slug protein and mRNA in Hep-2 cell line. The observed repression of Slug expression in the Hep-2 cell line by S100A4 may represent a mechanism for the inhibition of Slug function in laryngeal carcinoma.

The effect of S100A4 and Slug knockdown on cisplatin sensitivity in Hep-2 cell line seemed to depend on the degree of intrinsic S100A4 and Slug levels. To investigate the molecular mechanism by which S100A4 contributes to chemoresistance, we studied the effect of S100A4 knockdown on Hep-2 cell viability and apoptosis. We used siRNA for this analysis as we could achieve

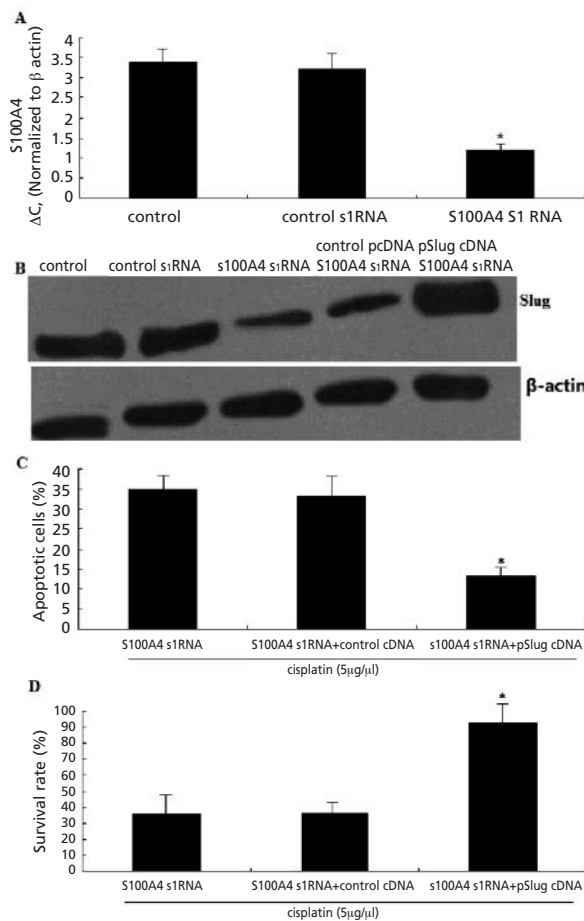


Figure 3. Knockdown of S100A4 promotes cisplatin-induced cytotoxicity by suppressing Slug. **A**, Hep-2 cells were transfected with S100A4-specific siRNA for 48 hs, Slug mRNA expression was detected by quantitative reverse transcription-PCR assay. **B**, Hep-2 cells were transfected with S100A4-specific siRNA for 48 hs, slug protein expression was detected by Western blotting. **C**, S100A4 siRNA transfected Hep-2 cells were transfected with the full coding region of human Slug (pSlug) for 48 hs, then the cells were subjected to cisplatin (5 $\mu\text{g}/\mu\text{l}$), the number of viable cells was quantified using the MTT assay over a period of 3d. **D**, S100A4 siRNA transfected Hep-2 cells were transfected with the full coding region of human Slug (pSlug) for 48 hs, then the cells were subjected to cisplatin (5 $\mu\text{g}/\mu\text{l}$), the number of apoptotic cells was quantified using the flow cytometry assay over a period of 3d. Vs S100A4 siRNA, * $p < 0.05$.

almost complete knockdown of the S100A4 transcript and protein by this method. This analysis showed that S100A4 or slug knockdown alone neither result in the reduction in cell viability and nor increased apoptotic cells in Hep-2 cells over 3 days. However, S100A4 or slug knockdown in Hep-2 cells increased cisplatin-induced cytotoxicity. Our observation also showed that S100A4 knockdown in Hep-2 cells decreased Slug level. However, when the S100A4 siRNA transfected Hep-2 cells were transfected with the full coding region of human Slug to restore the Slug level, the increase in apoptotic cells induced by S100A4 knockdown was significantly reduced, and the survival rate was significantly increased, which indicates that slug may be contributing to this effect.

Conclusions

The data presented in this article describe a novel role for S100A4/Slug signal in enhancing chemoresistance in laryngeal carcinoma through the suppression of apoptosis. This is an important finding in the context of laryngeal carcinoma for which all chemotherapeutic strategies have provided limited benefit. Further studies are now required to establish whether S100A4 can function as a survival factor in other cancer types as well as delineating the precise molecular mechanisms that mediate this activity.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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