Int J Clin Exp Pathol 2017;10(6):6512-6519
www.ijcep.com /ISSN:1936-2625/IJCEP0050192

Original Article

Inhibition of STAT3 with shRNA enhances the chemosensitization of cisplatin in laryngeal carcinoma stem cells

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Received February 6, 2017; Accepted April 25, 2017; Epub June 1, 2017; Published June 15, 2017

Abstract: Laryngeal carcinoma accounts for relatively high mortality rate due to therapy resistance and disease relapse. Increasing evidences have shown that recurrence and drug resistance are attributed to the existence of cancer stem cells (CSCs). Signal transducer and activator of transcription 3 (STAT3) is an important CSC-related molecule. In this study, we studied whether knockdown of STAT3 enhances cisplatin-sensitivity of laryngeal CSCs. We found that expression of STAT3 was elevated in Hep2-CSCs compared with Hep2 cells. Knockdown of STAT3 could induce apoptosis, reduce cell proliferation and tumorigenicity. In addition, knockdown of STAT3 remarkably enhanced the antitumor effect of cisplatin, indicating combination between cisplatin and shRNA targeting STAT3 may become a potential clinical approach for laryngeal carcinoma treatment.

Keywords: Laryngeal carcinoma, cancer stem cell, signal transducer and activator of transcription 3, apoptosis, RNA interference

Introduction

Laryngeal carcinoma is one of the most malignant tumors worldwide. Despite medical advances in early detection and treatment, laryngeal carcinoma still accounts for relatively high mortality rate due to its malignant nature, therapy-resistance and disease relapse [1]. Stem cells play many important roles in physiological and pathologic process [2]. The most common cause is attributed to the existence of a rare population of cells with stem cell-like properties, termed cancer stem cells (CSCs) or tumor-initiating cell (TIC), which contributes significantly to tumor progression, recurrence, drug resistance and metastasis [3]. Signal transducer and activator of transcription 3 (STAT3) is a transcription factor. STAT3 signaling plays a vital role in embryonic stem cell (ESC), which is required and sufficient to maintain the capacity of self-renewal and differentiation [4]. Recent evidence has also shown vital roles of STAT3 in promoting CSCs and progression of various tumors [4-7]. For example, knockdown of STAT3 efficiently decrease CSCs frequency [7]. Recent elegant data also indicated the STAT3 pathway agonist induced a significant increase of cancer stem-like population. Thus, developing agents capable of compromising CSCs capacity are urgently needed.

Cisplatin is the most commonly used chemotherapeutic drug for the treatment of laryngeal carcinoma as a single agent or in combination with other agents. Combination of cisplatin with other chemotherapy usually improves survival of laryngeal carcinoma patients [8]. However, these combinations also increase toxicities to normal tissues [9]. Therefore, the development of new therapeutic strategies aimed at enhancing the chemosensitivity of laryngeal carcinoma as well as reducing the toxicities of chemotherapy is required.

In this study, we found that blocking STAT3 expression suppressed tumorigenicity of laryngeal CSCs *in vitro* and *in vivo*. In addition, knockdown of STAT3 expression can sensitize laryngeal squamous cell carcinoma to cisplatin.

These findings not only reveal a vital role STAT3 in laryngeal cancer, but also highlight that combination between cisplatin and shRNA targeting STAT3 may become a potential clinical application for treating laryngeal carcinoma.

Materials and methods

Plasmids and cell culture

STAT3 shRNA expression plasmids were purchased from MISSION shRNA at Sigma-Aldrich (St Louis, MO). All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 10% fetal bovine serum (FBS). Hep-2 cells were collected and rinsed with phosphate-buffered solution (PBS). The number of dissociated cells was counted, then treated with fluorochrome-conjugated CD44+ antibody for 30 minutes at 4°C and protected from light. Once completed, cells were then washed and analyzed using a flow cytometer. The CD44+ and CD44- cells were sorted by the fluorescence-activated cell sorting (FACS) technique and the proportion of CD44+ cells were recorded. The CD44+ cells were further treated with PBS containing fluorochrome-conjugated ALDH+ antibody for 30 minutes at 4°C. Once completed, cells were washed and sorted by FACS and analyzed for the proportion of ALDH+ and CD44+ CSCs. For establishing stable transfectants with knockdown of STAT3 expression, Hep2-CSCs were transfected with STAT3 shRNA; stable clones were selected with puromycin (300 ng/ml) for 4 weeks.

Quantitative real-time PCR

Total RNA was isolated using RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Specific quantitative real-time PCR experiments were performed using SYBR Green Power Master Mix following manufacturer's protocol (Applied Biosystems). GAPDH served as a reference gene to normalize STAT3 genes. The GAPDH and STAT3 were amplified using the folowing primer sequences, respectively: GAPDH, forward 5'-GGTGGTCTCCTCTGACTTCAACA-3' and reverse 5'-GTTGCTGTAGCCAAATTCGTTGT-3'; STAT3, forward 5'-CTGGTGTCTCCACTGGTCTATCT-3' and reverse 5'-AAACTTGGTCTTCAGGTATGGG-3'.

MTT assay

The effect of shSTAT3, cisplatin or the shSTAT3/cisplatin combination on Hep2-CSCs proliferation was measured by MTT assay. 20 ul MTT was added to each well of 96-well plate and cells were incubated for 4 h at 37°C. After incubation, 150 µl DMSO was added to develop coloration. The plates were swirled gently in the dark for 2 h at room temperature. To observe the viability of cells, the absorbance values at 490 nm of each well was measured. Data were obtained from triplicate wells per condition and representative of at least three independent experiments.

Flow cytometry analysis

Cell apoptosis detection was performed with Annexin V/PI double staining. Cells were harvested by 0.25% trypsin, washed twice with PBS, followed by being resuspended in 250 µl of binding buffer, adjusted to 1×10^6/ml. Staining solution containing Annexin V/FITC and propidium iodide was added in cell suspension. After incubation in the dark for 30 min, the cells were analyzed by flow cytometry (FACSAria, Becton Dickinson, USA).

Invasion assay

Invasion assays were performed in Boyden chambers with coated Matrigel as instructed by the manufacturer (BD biosciences, San Jose, CA). The invasive cancer cells were stained with crystal violet and visualized with a microscope. All experiments were performed at least twice in triplicate. Statistical analysis was performed using the Student's t test; a p value of <0.05 was considered significant.

Xenograft studies

All animal studies were carried out according to the Guide for the Care and Use of Laboratory Animals. The research protocol was approved by the Animal Ethics Committee of Hangzhou Normal University. Nude mice were injected subcutaneously in the right back area with 5×10^6 cells in 100 µl of PBS. When tumors reached about 150 mm^3, animals were randomly grouped (5 animals/group) and given cisplatin (intraperitoneal injection) 10 mg/kg daily. Drug toxicity indexes such as weight loss, behavior change and feeding patterns were continuously observed during the whole treatment. The tumors were harvested and weighed at the experimental endpoint, and the masses of tumors Data were analyzed using the

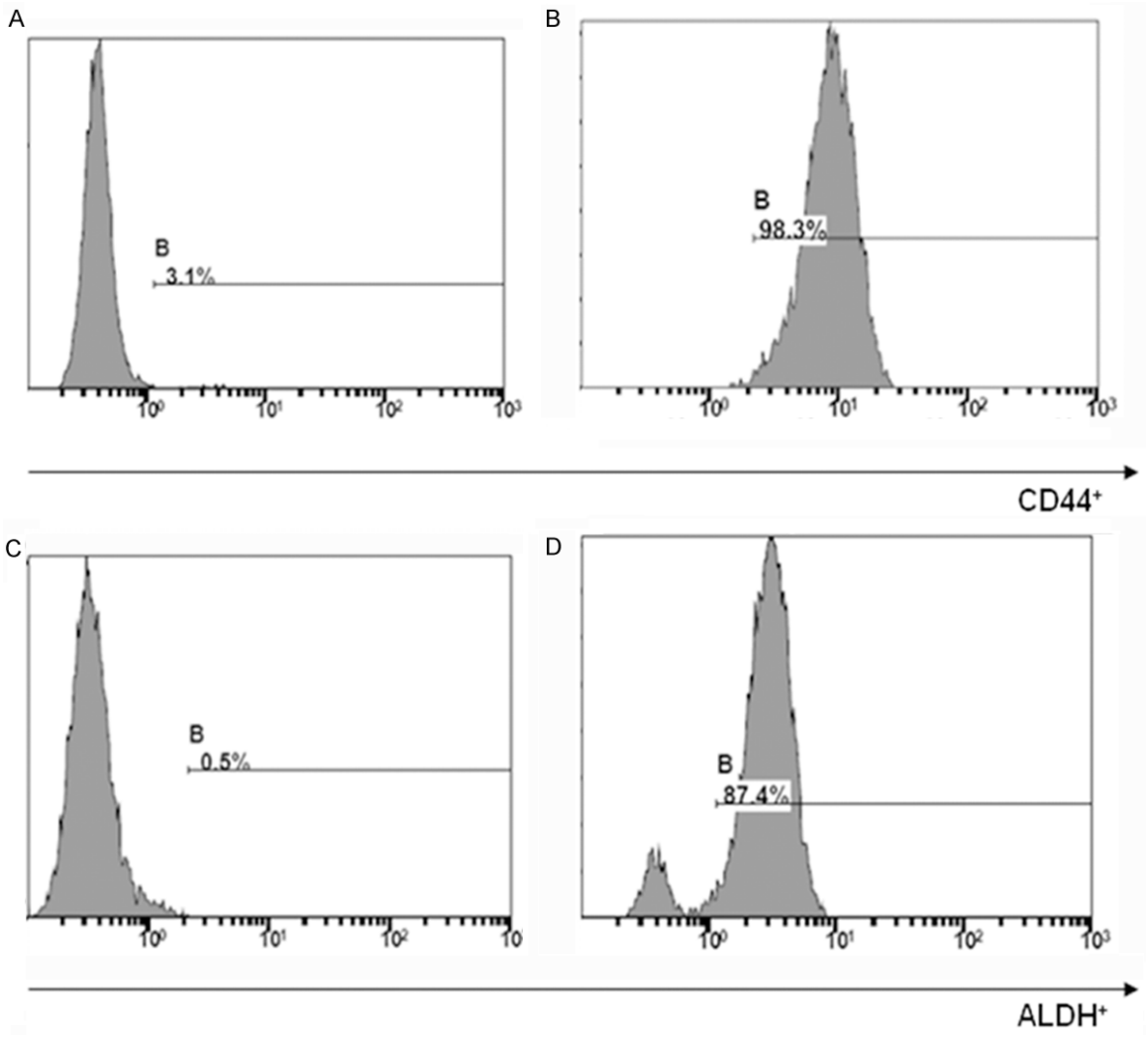

Figure 1. Isolation and identification of cancer stem cells ($CD44^+/ALDH^+$) in Hep2 cell line. Population of $CD44^+$ (A and B) and $ALDH^+$ (C and D) were isolated and identified by fluorescence activator cell sorting (FACS).

Student's t-test; a p value <0.05 was considered significant.

Results

Isolation and identification of cancer stem cells ($CD44^+/ALDH^+$) in Hep2 cell line

In order to isolate and identify the CSC population in Hep2 cell line, we used stem cell markers CD44 and ALDH with fluorescence activator cell sorting (FACS) to separate and count the number of cells expressing them. We observed only 3.1% CD44 positive cells were detected in Hep2 cell line (**Figure 1A**). After sorting, the percentage of CD44 positive cells was very high (98.3%) (**Figure 1B**). Subsequently, we found only 0.5% ALDH positive cells existed in the population of CD44 positive cells (**Figure 1C**). After sorting, there were 87.4% ALDH positive cells in the population of $CD44^+/ALDH^+$ cells (**Figure 1D**).

Elevated STAT3 expression is associated with Hep2-CSCs

Recent studies showed that STAT3 expression correlated with cancer stem cell. To identify the relationship between STAT3 expression and Hep2-CSCs, we examined the mRNA level of STAT3 both in Hep2 cells and Hep2-CSCs by real-time PCR. We found that expression of STAT3 was high in Hep2-CSCs compared with Hep2 cells, indicating STAT3 expression is positively correlated with Hep2-CSCs.

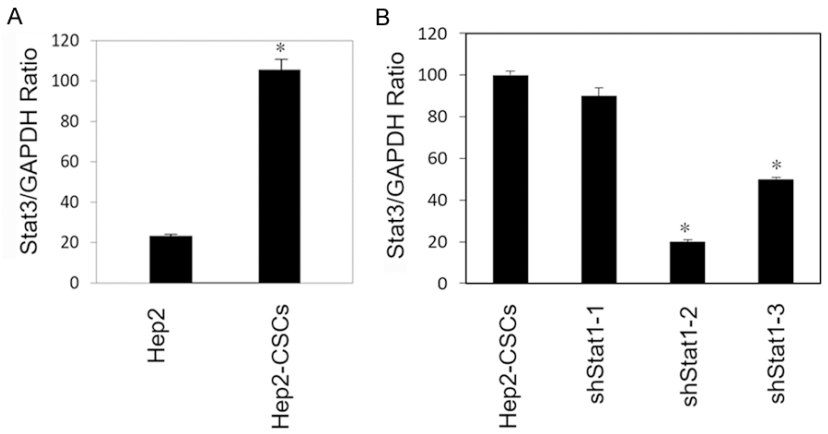

Figure 2. Elevated STAT3 expression is associated with Hep2-CSCs. A. The mRNA level of STAT3 in Hep2-CSCs was quantified by real-time PCR. *P<0.05, compared to Hep2. GAPDH served as a house-keeping gene. B. Stable shSTAT3 expressions were established in Hep2-CSCs, and the expression of STAT3 was quantified by real-time PCR. *P<0.05, compared to control.

Enhanced effect of knockdown of STAT3 on cisplatin mediated anti-proliferation and apoptosis

To examine whether STAT3 affects the growth of tumor cell proliferation and apoptosis, we established stable transfectants with shSTAT3 expression in Hep2-CSCs (**Figure 2**). We found that STAT3 expression was significantly downregulated in different stable transfectants with shSTAT3 expression. The inhibition effect of shSTAT3-2 was the best among them, and the subsequent experments were performed by using stable shSTAT3-2 transfectant. To test the effect of shSTAT3 on cell proliferation, Hep2-CSCs with stably expressing control vector, 10 μM cisplatin, shSTAT3 and shSTAT3 combined with 10 μM cisplatin treatments were examined by MTT assays. We observed cisplatin or shSTAT3 significantly inhibited Hep2-CSCs proliferation in a time dependent manner. Combined treatment dramatically enhanced the inhibition effect of shSTAT3 (**Figure 3A**). To evaluate shSTAT3-mediated apoptotic enhancement, Hep2-CSCs with stably expressing control vector, cisplatin, shSTAT3 and shSTAT3 combined with cisplatin treatment were subjected to FACS analysis. Cisplatin or shSTAT3 alone resulted in significant cell apoptosis, while shSTAT3 combined with cisplatin remarkably enhanced cell apoptotic rate compared with cisplatin alone. The percentage of apoptotic cells increased from 14.4% for control group to 36.2% for cisplatin and 65.9% for shSTAT3 combined with cisplatin (**Figure 3B**). These results indicate shSTAT3 enhance the effect of cisplatin on anti-proliferation and apoptosis.

Enhanced effect of knockdown of STAT3 on cisplatin mediated inhibition of colony-formation and cell invasion

We measured the *in vitro* tumorigenicity of these cells using soft-agar assay. We found Hep2-CSCs with cisplatin treatment have less colonies than their corresponding vector control; whereas shSTAT3 combined with cisplatin greatly decreased colony-formation compared the other groups (**Figure 4A** and **4B**). To identify if knockdown of STAT3 can enhance cisplatin-mediated tumor cell invasion, the tumor cell invasiveness was analyzed by invasion assay. We found cisplatin induced a 2-fold decrease in cell invasion, while cisplatin combined with knockdown of STAT3 caused even more remarkable decrease of cell invasion (5-fold) (**Figure 4C** and **4D**).

Combined with shSTAT3 enhanced the antitumor effect of cisplatin in vivo

To examine the tumorigenicity *in vivo*, we performed xenograft experiments in which we injected the right back area of nude mice as follows: (1) Hep2-CSCs with stably expressing control vector, (2) control vector with 10 mg/kg cisplatin daily for 10 d via i.p. injection, (3) shSTAT3, and (4) shSTAT3 combined with 10 mg/kg cisplatin daily for 10 d via i.p. injection. Mice were injected with 5×10^6 of cells. As shown in **Figure 5**, cisplatin or knockdown of STAT3 had a significant reduced size compared with vector control group. Interestingly, cisplatin combined with knockdown of STAT3 caused even more dramatic decrease of tumor size and weight (**Figure 5A** and **5B**). Together, these data indicate that knockdown of STAT3 enhances the antitumor effect of cisplatin *in vivo*.

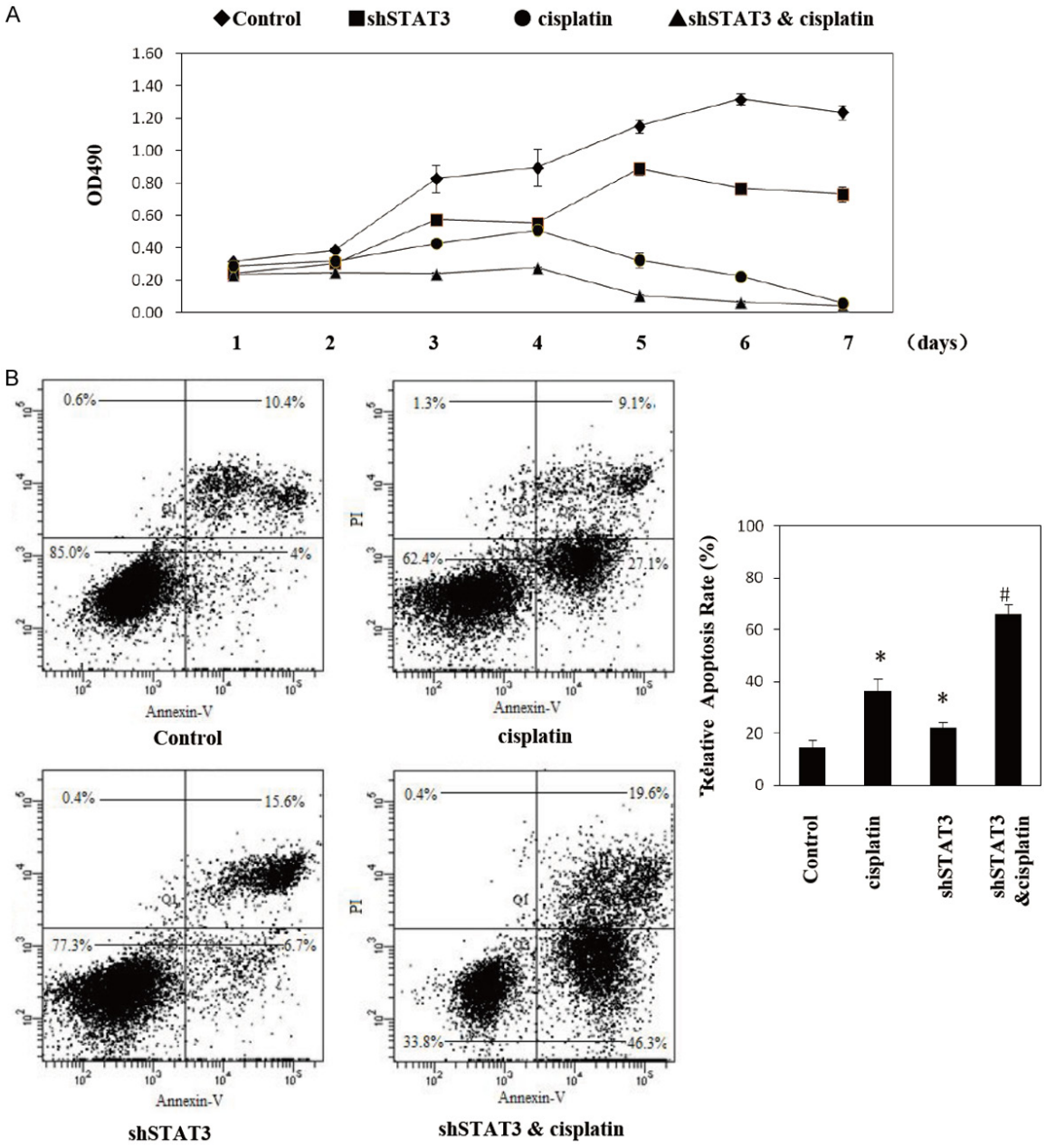

Figure 3. Combined effect of knockdown of STAT3 and cisplatin on cell proliferation and apoptosis. A. Cell proliferation of Hep2-CSCs with stably expressing control vector, shSTAT3 and shSTAT3 combined with cisplatin treatment were examined by MTT assays. Statistical analysis from three independent experiments with triplicate samples are shown on the bar graph. B. Cell apoptosis of Hep2-CSCs with stably expressing control vector, cisplatin, shSTAT3 and shSTAT3 combined with cisplatin treatment were tested. *P<0.05, compared to control, #P<0.01, compared to cisplatin.

Discussion

It has been shown that aggressiveness, drug resistance and recurrence in laryngeal cancer are attributed to CSCs through certain signaling pathways [3]. The activity and relative size of the CSC subpopulation within a tumor are regulated by inflammatory signals from the microenvironment [10]. The high expression of IL-6 and STAT3 are often found in therapy-insensitive cancers where they represent poor prognosis indicators [11]. Recently, accumulating evidence showed that STAT3 plays vital roles in regulating self-renewal and differentia-

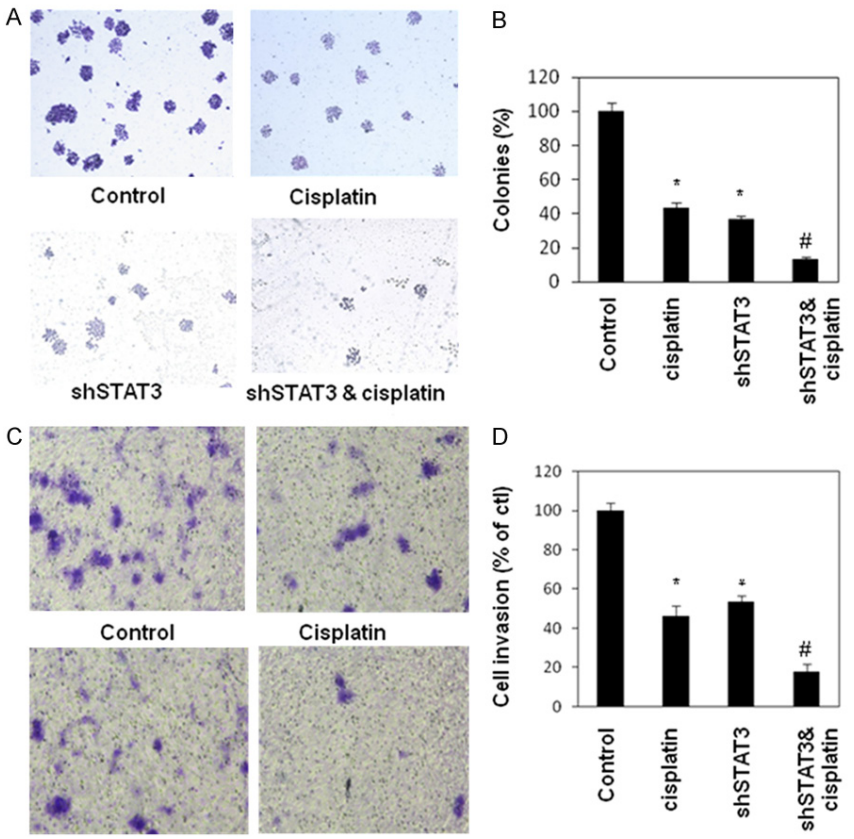

Figure 4. Combined effect of knockdown of STAT3 and cisplatin on colony-formation and cell invasion. A, B. Soft-agar assay was performed for stably expressing control vector, 10 μM cisplatin, shSTAT3 and shSTAT3 combined with 10 μM cisplatin treatment. Results were shown from three independent experiments with duplicates. C, D. The invasiveness of cells with stably expressing control vector, 10 μM cisplatin, shSTAT3 and shSTAT3 combined with cisplatin treatment was analyzed with a modified Boyden Chamber invasion assay as described in the Materials and Methods. The percentage of invasive cells is shown on the bottom panel (mean ± SD in three separate experiments). *P<0.05, compared to control, #P<0.01, compared to cisplatin.

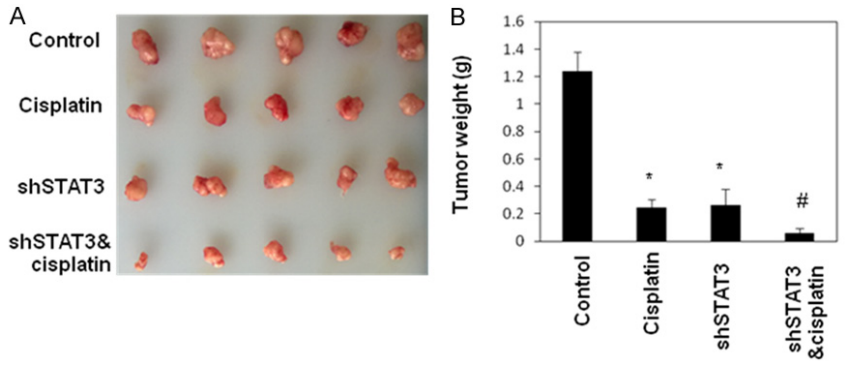

Figure 5. Combined antitumor activity of shSTAT3 and cisplatin on xenograft model. Nude mice were randomly assigned to four groups as follows: Hep2-CSCs with stably expressing control vector, control vector with 10 mg/kg cisplatin daily for 10 d via i.p. injection, shSTAT3, and shSTAT3 combined with 10 mg/kg cisplatin daily for 10 d via i.p. injection. A. A photograph of the tumors is shown. B. Tumor weight were measured and recorded. *P<0.05, compared to control, #P<0.01, compared to cisplatin.

tion of CSCs [12-17]. In this study, we successfully constructed stable transfectants with shSTAT3 expression that efficiently blocked STAT3 expression in Hep2-CSCs. We found that knockdown of STAT3 could induce apoptosis, reduce cell survival and tumorigenicity. These findings not only verify the vital role STAT3 in sustaining its "stemness" of laryngeal cancer, but also highlight that shRNA targeting STAT3 may have potential to become a powerful tool by its high efficiency, specificity and low toxicity.

Cisplatin is widely used in the treatment of laryngeal carcinomas [9]. Many initially responsive patients relapse within the first year of treatment because of cisplatin-resistance [18]. One strategy to overcome chemo-resistance is in combination with sensitizing agents. It's encouraging that cisplatin combined with mo-lecularly targeted agents, such as EGF inhibitor has exhibited reduced toxicities [19]. In our study, the cell proliferation was inhibited significantly in a time dependent manner when cisplatin and shRNA targeting STAT3 were used in combination. Consistent with these observations, this combination can remarkably induce Hep2-CSCs

apoptosis. To better characterize the observed synergic effect, tumor xenograft study was performed when shRNA targeting STAT3 and cisplatin were used individually or in combination. Combination treatment dramatically inhibited tumor growth compared with the single treatment, suggesting shRNA targeting STAT3 can sensitize xenografted laryngeal squamous cell carcinoma to cisplatin.

In summary, our study highlights the importance of shRNA targeting STAT3 in determining tumorigenicity of laryngeal carcinoma. Cisplatin-sensitivity of Hep2-CSCs can be enhanced by knockdown of STAT3 using shRNA targeting STAT3. Thus, combination between cisplatin and shRNA targeting STAT3 may become a potential clinical application for treating laryngeal carcinoma.

Acknowledgements

This work was supported by grants from Zhejiang Provincial Natural Science Foundation of China (LY12H13004, Y2100578), Shandong Provincial Natural Science Foundation (ZR2015JL027), Medicine and health care in Zhejiang province science and technology plan (2013ZDA018), Roadmap for Science and Technology Development of Hangzhou (20120533Q31) and National Science Foundation of China (NSFC81571512).

Disclosure of conflict of interest

None.

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