
Effects of Peritumoral Nanoconjugated Cisplatin on Laryngeal Cancer Stem Cells

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Objectives/Hypothesis: To evaluate the efficacy of peritumoral hyaluronic acid (HA)-cisplatin therapy in a murine model of laryngeal squamous cell carcinoma and to evaluate its effect on cancer stem cells (CSCs).

Study Design: An orthotopic murine study utilizing University of Michigan squamous cell carcinoma-12 (UMSCC-12) laryngeal cancer cells was conducted in randomized controlled fashion with three treatment arms: saline, systemic cisplatin, and peritumoral HA-cisplatin.

Methods: UMSCC-12 laryngeal cancer cells were inoculated into the buccal mucosa of athymic nude mice followed by weekly treatment with saline, systemic cisplatin, or peritumoral HA-cisplatin for 3 weeks. Tumor response and animal weight was monitored and change in CD44 proportion was analyzed ex vivo.

Results: HA-cisplatin demonstrated superior antitumor efficacy and greater reduction in CD44 positivity on ex vivo analysis.

Conclusions: Peritumoral nanoconjugated HA-cisplatin provides superior antitumor efficacy compared to standard cisplatin therapy in an in vivo laryngeal cancer model. There was also selective targeting of CD44+ cancer cells with HA-cisplatin. This therapeutic strategy could represent the first selective laryngeal CSC-targeted therapy. Further preclinical investigation is warranted to evaluate its role for locally advanced head and neck cancer treatment.

Key Words: Larynx cancer, cancer stem cells, targeted therapy, CD44, hyaluronan nanoconjugate.

Level of Evidence: NA

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INTRODUCTION

More than 55,000 Americans are expected to be diagnosed with head and neck cancers in 2014, with a large proportion of these being squamous cell carcinomas.¹ Approximately 12,000 of these oral cavity, pharyngeal, and laryngeal cancer patients will die from their disease yearly. Although the incidence of this disease in the United States has been noted to be relatively stable, recent studies have shown the rates of oropharyngeal

cancers associated with human papillomavirus to be rising.¹ These findings suggest that head and neck cancer will continue to be a prominent malignancy for treatment our in national healthcare cancer mission.

Historically, radiation therapy alone for patients with unresectable head and neck squamous cell carcinoma (HNSCC) provided unsatisfactory 5-year survival and recurrence rates. As a result, concurrent radiation and chemotherapy has now become the standard for adjuvant therapy after surgical ablation of the primary tumor and definitive treatment in select cases. Platinum-based chemotherapy, namely cisplatin, administered systemically via intravenous (IV) route remains a first-line agent due to its radiosensitizing and cytotoxic effects.² Cisplatin therapy, however, continues to have significant limitations. Besides its known toxicities to the kidney, peripheral nerves, and hearing, as a systemically delivered agent it has poor penetration into the locoregional lymphatics. Only a very small fraction of the systemic dose of the drug will be taken up into the lymphatics and lymph nodes, which may in part be due to monodirectional lymphatic flow and pressure gradients in these channels.³ This is a critical factor in patients with locally advanced disease where lymph node metastases are a frequent occurrence. Another major limitation is that HNSCC has also shown marked resistance to cisplatin in up to 40% of patients,⁴ which has important implications, as any efforts to overcome this resistance by increasing dosage has severe limitations due to the dose-dependent toxicity

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of the drug.⁵ Finally, cisplatin lacks specificity in targeting cancer stem cells (CSCs), considered by many to be the primary mediators of treatment failure.⁶ Thus far, targeted therapy against CSCs⁷ is a relatively unexplored but critically important area of study that needs further investigation.

Hyaluronic acid (HA) is an endogenous polysaccharide, with alternating D-glucuronic acid and N-acetyl D-glucosamine units, found in the extracellular matrix of connective tissues. In previously published work,⁸ there were significantly increased lymphatic tissue concentrations of cisplatin and reduced organ toxicities with peritumoral injections of cisplatin conjugated to nanoscopic (25–100 nM) particles of HA (HA-cisplatin) compared to systemically delivered standard cisplatin therapy. In related studies investigating this drug in orthotopic murine models, HA-cisplatin was found to have significantly higher anticancer efficacy *in vivo* relative to conventional IV cisplatin therapy in HNSCC xenografts implanted in the buccal mucosa of the subject mice.^{3,9}

Moreover, HA is also a highly specific ligand for the CD44 surface receptors,¹⁰ and CD44 is described as a cell surface marker specific for CSCs in HNSCC. In fact, CSCs were first successfully isolated from HNSCC cell lines utilizing CD44 expression.¹¹ The interactions between these two molecules, however, are yet to be investigated in the setting of stem cell–targeted therapy in HNSCC. Thus, the objectives of this study were to evaluate the efficacy and toxicity of peritumoral HA-cisplatin therapy compared to standard systemic cisplatin *in vivo* in a laryngeal cancer xenograft model via orthotopic tumor implantation, and to evaluate the effect of HA-cisplatin on the CD44 positive (+) HNSCC tumor population *ex vivo*.

MATERIALS AND METHODS

Cell Culture

University of Michigan Squamous Cell Carcinoma-12 (UMSCC-12) human laryngeal cancer cells were grown and maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Sigma-Aldrich), and 1% combination of penicillin and streptomycin (Sigma-Aldrich) in a 37°C humidified atmosphere of 5% CO₂ in air. To utilize the cells for the various experiments, they were trypsinized using 0.25% trypsin (Sigma-Aldrich), collected, and counted with a hemocytometer to determine the number of viable cells.

In Vitro CD44 Analysis

In vitro analysis was performed prior to conducting the *in vivo* study to ensure that the UMSCC-12 cell line had a relatively high CD44+ proportion of cells. To determine the CD44+ proportion, the cells were first trypsinized, collected, rinsed in 1× phosphate-buffered saline (PBS), and placed in single-cell suspension. They were then treated with fluorochrome-conjugated CD44 antibody (BD Biosciences, San Diego, CA) for 30 minutes on ice. Once completed, the cells were rinsed again in 1× PBS and resuspended before being analyzed via flow cytometry (Beckman Coulter CyAn ADP analyzer; Beckman Coulter, Brea, CA).

Cell Viability Assay

To examine the *in vitro* effect of HA-cisplatin and standard cisplatin on the viability and proliferation of UMSCC-12 cells, a standard 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay was performed. Cells were seeded in 96-well plates at a density of 1,000 cells per well. After a 24-hour incubation period, HA-cisplatin and cisplatin were added to each well in varying concentrations and then incubated for 72 hours. Cell viability was then quantified by the colorimetric CellTiter96 Aqueous MTS assay (Promega, Fitchburg, WI) at 490 nm on a BioTek Synergy 2 plate reader (BioTek, Winooski, VT) according to the manufacturer's instructions.

In Vivo Tumor Model

UMSCC-12 cells were allowed to proliferate in cell culture as previously described and then inoculated in the buccal mucosa of 33 athymic nude female mice 4 to 6 weeks in age (Harlan Laboratories, Inc., Indianapolis, IN). The cells were prepared in 1× PBS solution at a concentration of 1×10^6 cells per 100 μ L. Tumor inoculation was performed under isoflurane anesthesia using a 25-gauge needle. Tumor size and body weight were then measured three times weekly with a digital caliper. Tumor volume measured in cubic millimeters was calculated using the following equation: Tumor Volume = $(\pi/6) \times (\text{Width})^2 \times \text{Length}$.

Body weight was measured in grams using a standard digital scale. Once tumor volumes reached 50 mm³, the mice were randomized into three treatment arms with 11 mice in each arm: control, systemic cisplatin, and peritumoral HA-cisplatin. Subjects were treated weekly at 50% maximum tolerated dose (MTD) for the two treatment arms and with PBS for the control arm for 3 weeks. Pharmaceutical-grade cisplatin was utilized for both the systemic group and in the HA-cisplatin group, with the latter nanoconjugated formulation synthesized as described in previously published work.¹² Treatments in the control and standard cisplatin arms were administered intraperitoneally, and treatments in the HA-cisplatin group were administered subcutaneously around the tumor site. The MTD level used was 10 mg/kg per week as previously reported in studies where intraperitoneal cisplatin was dosed in mice.^{13,14}

Once the treatment period concluded, three mice from each arm, designated as the early post-treatment group, were then euthanized, the tumors were harvested, and *ex vivo* CD44 analysis of the samples were performed. The remaining subjects were then monitored for 9 weeks post-treatment for tumor size and body weight. At the end of the study, four mice from each arm were analyzed for CD44+ cell proportion in the tumors *ex vivo*.

Ex Vivo CD44 Analysis

Harvested tumors were prepared for CD44 analysis via either Western blotting or flow cytometry. For Western blot analysis, proteins were collected, quantified, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and electrotransferred onto a Hybond nitrocellulose membrane as described in a prior study.¹⁵ Actin levels were assessed to ensure relatively equal protein loading and transfer among the tumor samples. Primary mouse antibodies included anti-CD44 antibody (#5640; 1:1,000; Cell Signaling Technology, Beverly, MA) and anti-actin antibody (MAB1501; 1:50,000; EMD Millipore, Billerica, MA). Secondary anti-mouse antibody staining was then applied at 1:5,000 for anti-CD44 antibody and 1:10,000 for anti-actin antibody. Both for the early post-

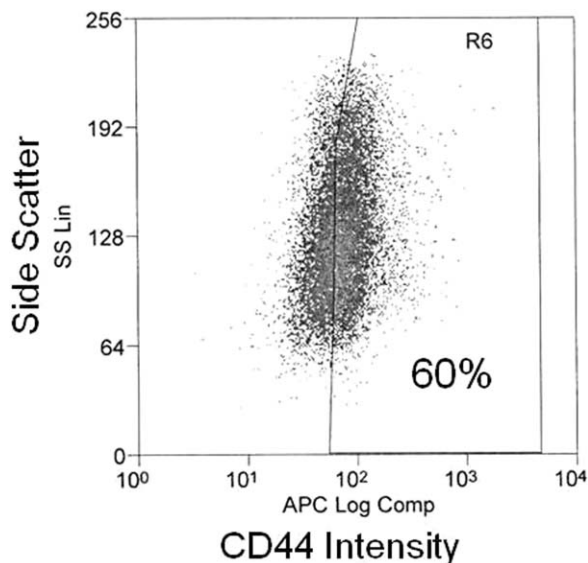


Fig. 1. One sample of CD44+ cell proportion in UMSSC-12 cell culture population in vitro as determined by flow cytometry.

treatment and at the end of the study, tumor samples were analyzed via Western blot.

For flow cytometry analysis of CD44 proportion, the tumor samples were processed using a detailed tumor digestion protocol available via the University of Michigan Comprehensive Cancer Center Wicha Lab manual available online.¹⁶ The main digestive agent utilized was 10× collagenase/hyaluronidase (STEMCELL Technologies Inc., Vancouver, BC, Canada) diluted 1:10 in Medium 199 (Sigma-Aldrich). The resultant tumor cells were then converted to single-cell suspensions and treated with fluorochrome-conjugated CD44 antibody (BD Biosciences) and then analyzed via flow cytometry (Beckman Coulter CyAn ADP analyzer; Beckman Coulter) for CD44+ cell proportion. Of note, only the end-of-study tumor samples were large enough to be processed for flow cytometry and Western blotting.

Statistical Analysis

GraphPad Prism 6 (version 6.02; GraphPad Inc., San Diego, CA) was used to generate best-fit nonlinear dose response curves for half-maximal inhibitory concentration (IC₅₀) determination for the MTS assay data. Comparisons of differences between two means were determined by Student unpaired *t* test via the statistical functions of Microsoft Excel 2010 software (Microsoft Corp., Redmond, WA). Data are presented as mean values with error bars denoting standard deviation. The level of significance was set at *P* < .05.

RESULTS

In Vitro Analysis of UMSSC-12 Cell Line

Flow cytometry analysis showed the CD44+ proportion of cells in UMSSC-12 to be on average 61% ± 6% in this cell population when grown in vitro (Fig. 1). The relatively high proportion of CD44+ cells (60%) within this cell line validated its use in the in vivo model to ensure that a knockdown effect on CD44 expression could be clearly detected with drug treatment if such an effect occurred. To evaluate the cytotoxic effect of HA-cisplatin on UMSSC-12 cells, the cells were plated and treated

with increasing concentrations of HA-cisplatin as well as standard cisplatin for 72 hours with resultant cell viability determined by MTS assay (Fig. 2). A dose-dependent decrease in cell viability was seen with both drugs, demonstrating that conjugation to HA does not inhibit the cytotoxic effect of cisplatin (as previously described).¹³ GraphPad was used to determine the IC₅₀ values of the two drugs. IC₅₀ values were comparable between the two treatment drugs at 3.01 ± 0.59 μg/mL for cisplatin and 5.59 ± 0.76 μg/mL for HA-cisplatin (*P* = .3).

In Vivo Analysis of Efficacy and Toxicity

To assess the overall efficacy of HA-cisplatin in vivo, an orthotopic xenograft murine model was utilized. Three separate treatment arms were set in a randomized, controlled manner, and these included control, standard cisplatin, and HA-cisplatin, with 11 mice in each group from the start. After 3 weeks of treatment, an early post-treatment group of three mice from each arm were euthanized, and their tumors were analyzed for CD44+ proportion. The remaining mice were then monitored three times a week for tumor volume and body weight for nine weeks, at which point the study was concluded. HA-cisplatin showed superior antitumor efficacy compared to cisplatin as shown by tumor volumes at 4 weeks post-treatment (39.2 mm³ vs. 57.6 mm³, *P* = .02), but at end of the study the difference was not significant (77.5 mm vs. 105.6 mm³, *P* = .05) (Fig. 3). Tumor volumes in the control group demonstrated an exponential growth curve approximately 4 weeks after completion of treatment. The tumor volumes in both treatment arms, in contrast, were initially more stable, followed by a shallow linear growth rate. One study subject in the HA-cisplatin arm experienced a complete response (CR), and one had a sustained partial response (PR) (Fig. 4). None of the standard cisplatin mice had CR or PR. In evaluating toxicity, animal weights were found to be similar between the cisplatin and HA-cisplatin arms at 24.7 ± 0.69 g and 24.5 ± 0.81 g, respectively (Fig. 5).

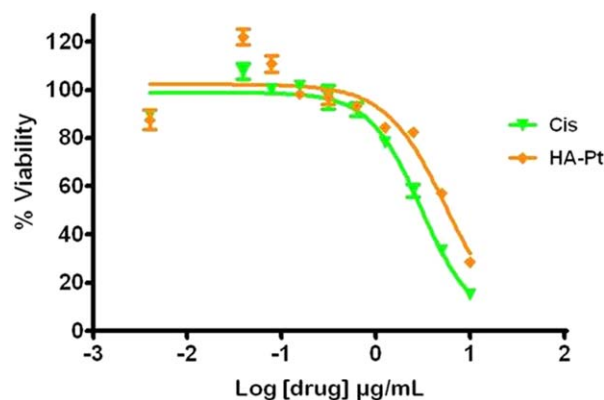


Fig. 2. Best-fit nonlinear dose response curves showing dose-dependent decrease in cell viability of UMSSC-12 cells with drug treatment. Cis = cisplatin; HA = hyaluronic acid; UMSSC-12 = University of Michigan Squamous Cell Carcinoma-12; HA-Pt = hyaluronic acid conjugated cisplatin. [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.]

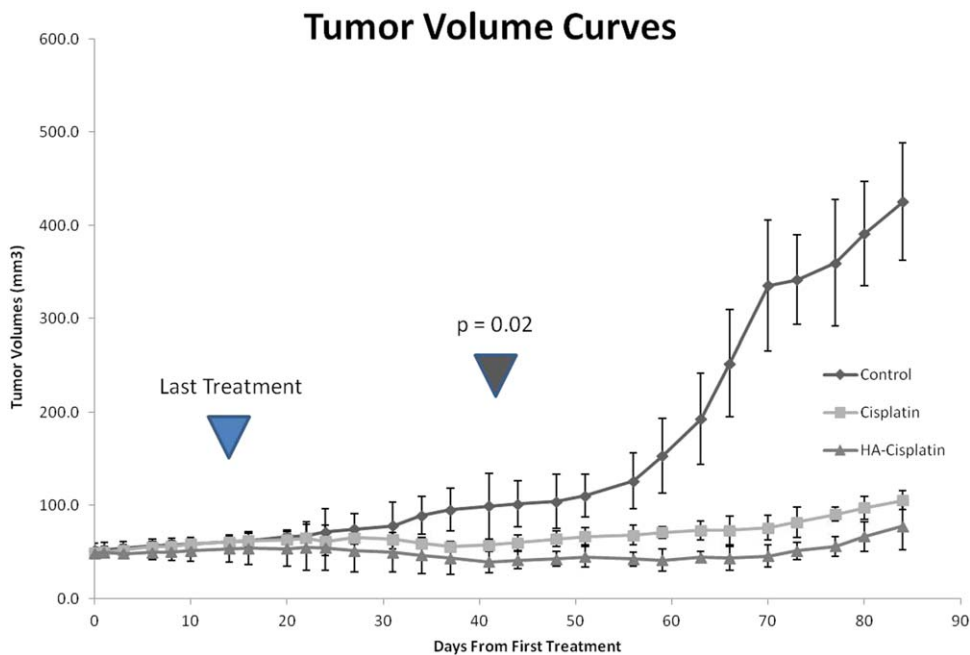


Fig. 3. Tumor response curves for the three treatment arms. HA = hyaluronic acid. [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.]

Ex Vivo Analysis of CD44

In the early post-treatment group, CD44 reduction was seen in both treatment groups, but to a greater degree with HA-cisplatin as demonstrated by Western blotting (Fig. 6). At the end of the study, four mice from each arm were then euthanized and tumors were analyzed for CD44+ proportion, both by flow cytometry and Western blotting (Figs. 7 and 8). Long-term durability of this CD44 knockdown effect was seen at the end of the study in the HA-cisplatin group compared to cisplatin (8.1% CD44+ vs. 23.9% CD44+, $P = .02$), which was similar to control (23.9% CD44+ vs. 24.8% CD44+, $P = .86$). The flow cytometry data were confirmed by the Western blot results, which clearly demonstrated reduced expression of CD44 in the HA-cisplatin group, which was maintained even 8 weeks after removal of drug therapy.

DISCUSSION

Concurrent chemoradiation therapy has lead to improved locoregional disease control compared to monotherapy and is currently the standard of care for locally advanced HNSCC patients following surgery. Even with

these advancements, however, survival rates for patients with HNSCC have improved little the last 3 decades. In efforts to improve outcomes, there have been several phase III trials that implemented cisplatin in combination with one or more drugs, such as 5-fluorouracil. Some studies showed promising results, but others showed only minimal improvement in disease-free survival and locoregional control compared to patients receiving radiation therapy alone.^{2,17,18}

This grim clinical picture of HNSCC supports the vital need for discovery and development of better treatments, and improved drug delivery over traditional systemic therapy administered intravenously. A locoregionally directed drug delivery, such as nanoconjugated HA-cisplatin via a weekly peritumoral injection, as was utilized in this study, may be an important treatment advance in a thus far stagnant paradigm of treatment for advanced HNSCC. Although localized therapy is already being used in the treatment of esophageal cancers, limb-limited melanoma with limb perfusion techniques, and hepatic cancers via transarterial chemoembolization techniques,^{19,20} this therapeutic strategy has yet to be clinically implemented in the treatment of HNSCC.



Fig. 4. Examples of subject mice. (A) A cisplatin mouse early after completion of treatment. (B) A hyaluronic acid-cisplatin mouse with partial response. (C) A control mouse showing uninhibited tumor growth. [Color figure can be viewed in the online issue, which is available at www.laryngoscope.com.]

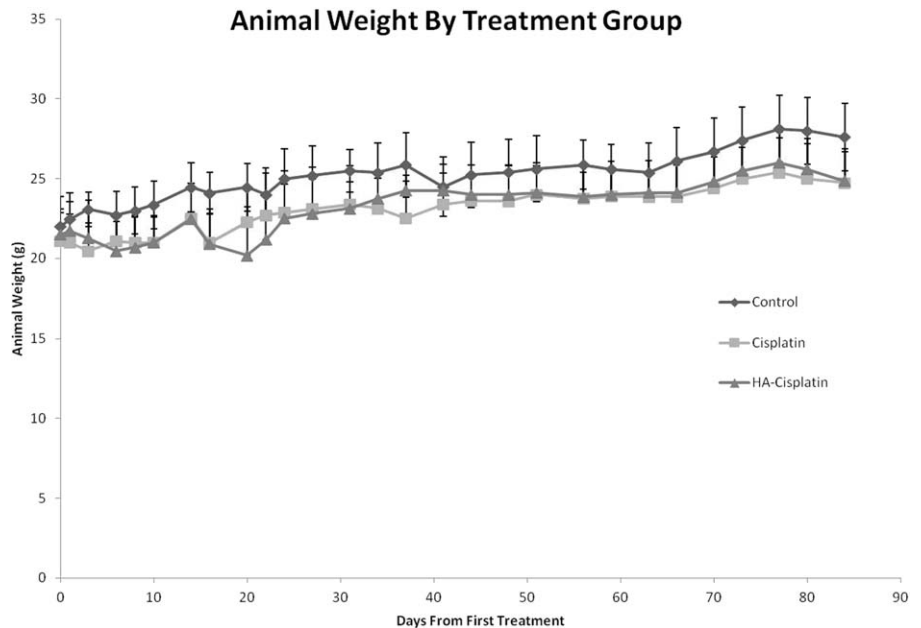


Fig. 5. Weights of animal subjects monitored throughout the study. HA = hyaluronic acid.

The current study demonstrates the improved *in vivo* efficacy of localized, peritumoral HA-cisplatin therapy compared to systemic cisplatin therapy, especially in the early post-treatment period. The difference in tumor volume between the two treatment groups, however, was not statistically significant by the end of the study. It is important to note, though, that one mouse in the HA-cisplatin arm had a complete response with no evidence of residual tumor, and one other had a sustained partial response at the end of the study. No mice in the standard cisplatin arm experienced such a response. But the overall efficacy of standard cisplatin in this study utilizing a laryngeal cancer cell line is not surprising given its proven efficacy clinically in the setting of advanced laryngeal cancer treatment. In fact, in some institutions, systemic cisplatin is used as a chemo-selection agent to determine candidacy for organ preservation therapy as an alternative to total laryngectomy in patients with advanced laryngeal squamous cell carcinoma.²¹

The natural progression of HNSCCs is to preferentially spread to the locoregional lymphatic basins. Hematogenous spread is less frequent than lymphatic spread in squamous cell carcinomas.²² Despite this, systemic IV

therapy has remained the standard modality of treatment. Locoregional peritumoral therapy delivers drug into the lymphatic channels, directly affecting the primary tumor and its associated lymphatic basins. This could prove to be the more effective strategy against locally advanced or recurrent disease in the future. The current study did not incorporate the treatment of nodal disease to the *in vivo* model, mainly due to the low *in vivo* metastatic potential of the UMSSC-12 cell line. Previously published studies on HA-cisplatin, however, demonstrated efficacy of peritumoral HA-cisplatin therapy even at the regional nodal basins.^{3,9} This suggests that HA-cisplatin could prove to be an effective therapy in locoregionally advanced HNSCCs in the human translational setting with potential to even improve survival rates in this subset of patients.

Solid tumors, including HNSCCs, normally consist of a heterogeneous population of cell types including tumor, stromal, and inflammatory cells.⁶ Among the tumor cells, CSCs represent a small subpopulation of cells that have the unique ability to self-renew through multiple generations, regenerate cells that have both tumorigenic and nontumorigenic properties, and sustain growth of the tumor.²³⁻²⁷ This subpopulation is responsible for most of the growth and spread of tumor, with the non-CSC population having limited to no growth potential.¹¹ Analogous to normal stem cells, CSCs can lie quiescent for periods of time and harbor mechanisms to protect against DNA and cellular damage.⁶ Given these properties, it is not surprising that CSCs have been shown to have resistance against chemotherapeutic agents and external beam radiation.^{28,29} An additional feature of CSCs is that they can be isolated from the other tumor cell types by distinctive cell surface markers, namely CD44 and ALDH in HNSCC. In fact, CSCs were first successfully isolated from HNSCC cell lines utilizing CD44 expression.¹¹ It is theorized that

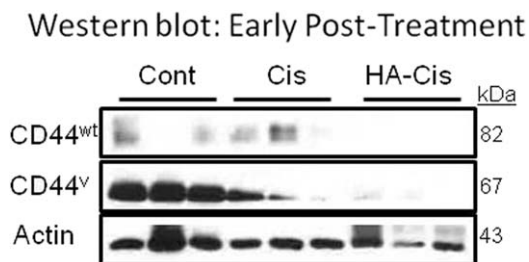


Fig. 6. Western blot from early post-treatment group demonstrating reduction of CD44 expression in the two treatment arms, but to greater degree in HA-cisplatin. Cis = cisplatin; HA = hyaluronic acid.

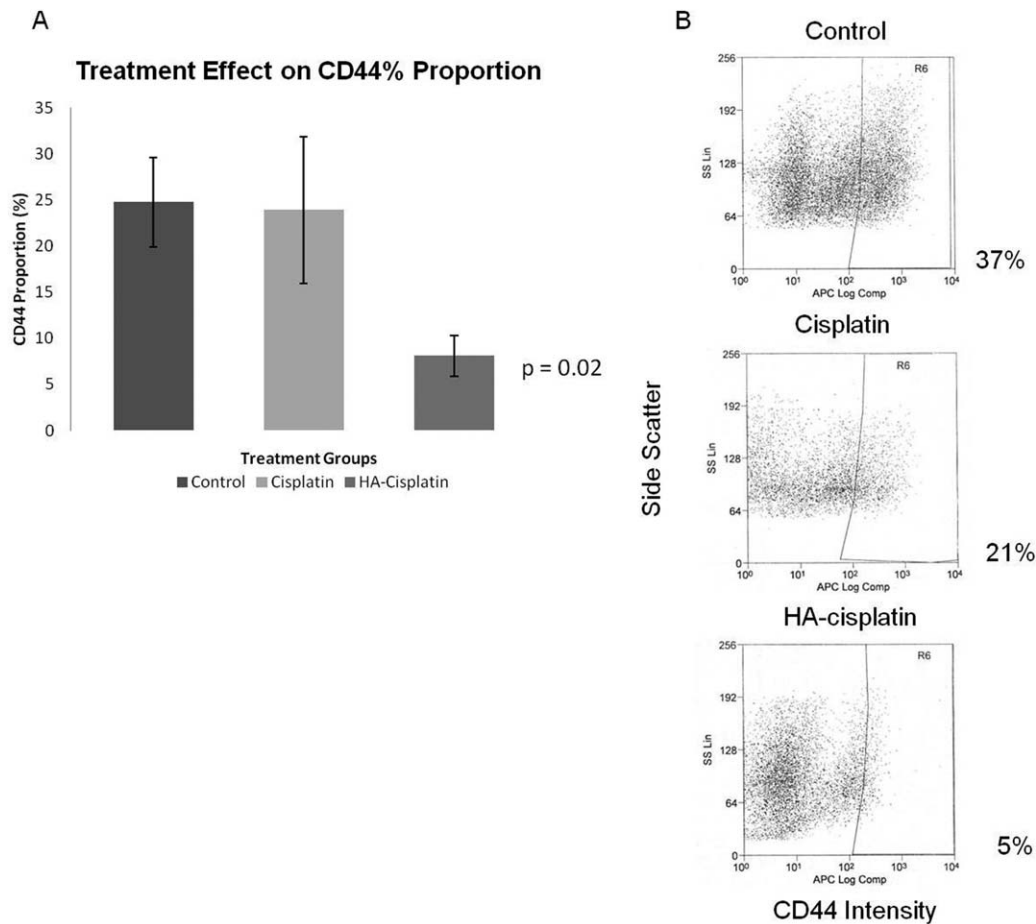


Fig. 7. (A) Flow cytometry data from end-of-study group demonstrating significant reduction of CD44 expression in the HA-cisplatin group. (B) Examples of flow cytometry diagrams from each group. HA = hyaluronic acid.

treatment failures are due to a failure in targeting and eliminating CSCs, resulting in tumor regrowth and resistance to therapy.³⁰

Coincidentally, HA nanoconjugation, which allowed for cisplatin to be administered via a peritumoral route, also provided the potential for HA-cisplatin to preferentially target the CD44⁺ subpopulation, and thus, the CSCs in a tumor. The results of the current study showed significant evidence of this selective CD44 targeting by HA-cisplatin. The early post-treatment tumors

showed decreased expression of CD44 in the HA-cisplatin-treated group compared to cisplatin-treated mice and controls according to Western blot analysis. The end-of-study tumors showed significant reduction in CD44⁺ cell proportion via flow cytometry in the HA-cisplatin treated populations, and these findings correlated with the Western blot analysis. It is important to note, however, that although decrease in CD44 positivity allows one to infer effective CSC-targeting, a more accurate way to determine this would incorporate assessing for both CD44 and ALDH positivity. Overall, these results provide the preliminary basis for further exploration and development of CSC-specific therapies.

Western blot: End-of-Study

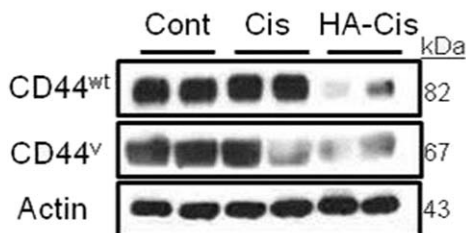


Fig. 8. Western blot from end-of-study group demonstrating reduction of CD44 expression in HA-cisplatin group. Cis = cisplatin; HA = hyaluronic acid.

CONCLUSION

Peritumoral nanoconjugated HA-cisplatin provides superior antitumor efficacy compared to standard cisplatin therapy in an in vivo laryngeal cancer xenograft model. Moreover, it may potentially target laryngeal CSCs as evidenced by its selective effect on CD44⁺ cells within a heterogeneous tumor population. These results provide support for further translational investigation of this treatment modality as a potential CSC-targeting agent for future clinical application to patients with locally advanced HNSCC.

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