Cucurbitacin I Suppressed Stem-Like Property and Enhanced Radiation-Induced Apoptosis in Head and Neck Squamous Carcinoma–Derived CD44\(^+\)ALDH1\(^+\) Cells

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**Abstract**

Head and neck squamous cell carcinoma (HNSCC) is a prevalent cancer worldwide. Signal transducers and activators of transcription 3 (STAT3) signaling is reported to promote tumor malignancy and recurrence in HNSCC. Cucurbitacins, triterpenoid derivatives, are strong STAT3 inhibitors with anticancer properties. Recent studies have shown aldehyde dehydrogenase 1 (ALDH1) to be a marker of cancer stem cells (CSC) in HNSCC. The aim of this study was to investigate the therapeutic effect of cucurbitacin I in HNSCC-derived CSCs. Using immunohistochemical analysis, we firstly showed that CD44, ALDH1, and phosphorylated STAT3 (p-STAT3) were higher in high-grade HNSCCs, and that triple positivity for CD44/ALDH1/p-STAT3 indicated a worse prognosis for HNSCC patients. Secondly, CD44\(^+\)ALDH1\(^+\) cells isolated from seven HNSCC patients showed greater tumorigenicity, radioresistance, and high expression of stemness (Bmi-1/Oct-4/Nanog) and epithelial-mesenchymal-transitional (Snail/Twist) genes as p-STAT3 level increased. Furthermore, we found that cucurbitacin I (JSI-124) can effectively inhibit the expression of p-STAT3 and capacities for tumorigenicity, sphere formation, and radioresistance in HNSCC-CD44\(^+\)ALDH1\(^+\). Notably, 150 nmol/L cucurbitacin I effectively blocked STAT3 signaling and downstream survivin and Bcl-2 expression, and it induced apoptosis in HNSCC-CD44\(^+\)ALDH1\(^+\). Moreover, microarray data indicated that 100 nmol/L cucurbitacin I facilitated CD44\(^+\)ALDH1\(^+\) cells to differentiate into CD44\(^−\)ALDH1\(^−\) and enhanced the radiosensitivity of HNSCC-CD44\(^+\)ALDH1\(^+\). Xenotransplant experiments revealed that cucurbitacin I combined with radiotherapy significantly suppressed tumorigenesis and lung metastasis and further improved the survival rate in HNSCC-CD44\(^+\)ALDH1\(^+\)-transplanted immunocompromised mice. Taken together, our data show that cucurbitacin I, STAT3 inhibitor, reduces radiosensitive, distant-metastatic, and CSC-like properties of HNSCC-CD44\(^+\)ALDH1\(^+\) cells. The potential of cucurbitacin I as a radiosensitizer should be verified in future anti-CSC therapy. *Mol Cancer Ther; 9(11); 2879–92.* ©2010 AACR.

**Introduction**

Head and neck squamous cell carcinoma (HNSCC), including oral squamous cell carcinoma, is the sixth most prevalent cancer worldwide and accounts for approximately 8% to 10% of all cancers in Southeast Asia (1, 2). In spite of improvements in the diagnosis and management of HNSCC, long-term survival rates have improved only marginally over the past decade (3). To increase the patient survival rate, investigations elucidating the mechanisms of tumorigenicity in HNSCC are urgently needed. Recently, Prince et al. showed that the purified CD44-positive (CD44\(^+\)) population of HNSCC cells possesses the self-renewing properties of cancer stem cells (4). Aldehyde dehydrogenase 1 (ALDH1), a cytosolic isoenzyme, is responsible for oxidizing intracellular aldehydes and contributing to the oxidation of retinol to retinoic acid in early stem cell differentiation (5, 6). Visus et al. further suggested that ALDH1A1 is a marker of HNSCC that distinguishes malignant from premalignant cells and is also an essential epitope for developing ALDH1A1-based vaccines for HNSCC therapy (7). Recently, ALDH1 has been shown to be a putative marker of cancer stem cells (CSC) in breast cancer, hepatoma, colon cancer, and HNSCC (5, 6). Importantly, CD44/ALDH1-coexpressing HNSCC cells display high tumorigenic and radioresistant properties and may serve as a reservoir for developing tumors and metastasis (8).

Signal transducer and activator of transcription 3 (STAT3), a transcription factor for cytokine signaling, is...
constitutively activated in prostate cancer, breast cancer, leukemia, multiple myeloma, brain tumors, and HNSCC (9–13). Bromberg et al. reported that STAT3 mutations induce cellular transformation and tumor formation in vivo and that activation of STAT3 signaling further inhibits p53 transcriptional activity, fulfilling the definition of an oncogene (14, 15). Oncogenic STAT3 activation leads to increased expression of downstream genes that suppress apoptosis (Bcl-xl), regulate cell cycle progression (p21, c-Myc, and cyclin D1), mediate cellular invasion (MMP-9), and modulate angiogenesis (VEGF; ref. 16). Persistent STAT3 activation is associated with mutations in epidermal growth factor (EGF) receptor (EGFR) and abrogates growth factor dependence and apoptosis in HNSCC cells (13). The activation of STAT3 in HNSCC has been suggested to serve as a prognostic indicator for tumor growth and malignant progression (17). Notably, recent reports have suggested that inhibition of STAT3 in cancer cell lines can significantly increase radiosensitivity and radiation-induced apoptosis and further suppress tumorigenicity as well as angiogenesis (18–20). However, whether STAT3 plays a role in maintaining the self-renewal and radioresistance in HNSCC-associated CSC is still an open question.

Due to the pivotal role of STAT3 in HNSCC and other cancers, numerous studies have focused on identifying safe and effective therapeutic agents that can abrogate constitutively active STAT3 signaling. Cucurbitacin I (also known as JSI-124), a natural cell-permeable triterpenoid compound, belongs to the cucurbitacin family of drugs isolated from various plant families, such as the cucurbitaceae and cruciferae. Cucurbitacins have been used as folk medicines for centuries due to their anti-inflammatory and analgesic effects. Recent studies have reported that cucurbitacin I potently inhibits cell growth via selectively repressing tyrosine phosphorylation of STAT3 in various human cancer cell lines (21). In this study, we show that the subset of CD44+ALDH1+ cells isolated from seven HNSCC patients presented cancer stem-like properties, radioresistance, and high levels of p-STAT3. According to microarray and bioinformatic analysis, the treatment of cucurbitacin I showed the potential to promote the CSC-like subset of CD44⁺ALDH1⁺ cells shift toward ALDH1⁺ and low-grade HNSCC cells. Furthermore, we investigated whether targeting STAT3 signaling with cucurbitacin I sensitized the HNSCC-derived CSC to radiation treatments. Our results show that cucurbitacin I inhibited the CSC-like properties, enhanced the radiosensitivity, and suppressed the lung metastatic ability of HNSCC-CD44⁺ALDH1⁺ cells in vivo.

Materials and Methods

Reagents

Cucurbitacin I (JSI-124; Supplementary Fig. S1) was purchased from Sigma Chemical Co. and dissolved in DMSO as a stock solution of 100 μmol/L. Cucurbitacin I was further diluted in culture medium to appropriate final concentrations just before use.

Isolation of CD44⁺ALDH1⁺ cell subsets from head and neck tissues

This research followed the tenets of the Declaration of Helsinki. All samples were obtained after patients provided informed consent. The study was approved by the Institutional Ethics Committee/Institutional Review Board of Taipei Veterans General Hospital. The dissociated cells derived from the samples of HNSCC patients were suspended at a concentration of 1 × 10⁷/mL in 37°C DMEM with 2% FCS. We then identified CD44-positive and/or ALDH1-positive cells in the HNSCC cell samples using CD44 antibody (phycoerythrin conjugated, BioLegend) and/or the Aldefluor assay (StemCell Technologies), followed by fluorescence-activated cell sorting analysis (FACS). CD44⁺ALDH1⁺ cells were cultured in serum-free DMEM/F12 (GIBCO) medium supplemented with N2 supplement (R&D), 10 ng/mL human recombinant basic fibroblast growth factor (bFGF; R&D), and 10 ng/mL EGF (22, 23). For evaluation of cell proliferation, cells were seeded on 24-well plates at a density of 2 × 10⁴ cells/well in medium, followed by the methyl thiazole tetrazolium assay (MTT assay, Sigma-Aldrich Co.).

Irradiation and clonogenic assay

γ-Radiation was delivered by a Theratronic cobalt unit T-1000 (Theratronic International, Inc.) at a dose rate of 1.1 Gy/minute (source-to-surface distance = 57.5 cm). For clonogenic assay, cells were exposed to different radiation doses (0, 2, 4, 6, 8, and 10 Gy). After incubation for 10 days, colonies (>50 cells/colony) were fixed and stained for 20 minutes with a solution containing crystal violet and methanol. Cell survival was determined by means of colony formation assay. The plating efficiency (PE) and survival fraction (SF) were calculated as follows: PE = (colony number/inoculating cell number) × 100%; SF = colonies counted/ (cells seeded × PE/100).

Microarray analysis and bioinformatics

Total RNA was extracted from cells using Trizol reagent (Life Technologies) and the Qiagen RNAeasy (Qiagen) column for purification. Total RNA was reverse-transcribed with Superscript II RNase H-reverse transcriptase (Gibco BRL) to generate Cy3- and Cy5-labeled (Amersham Biosciences Co.) cDNA probes for the control and treated samples, respectively. The labeled probes were hybridized to a cDNA microarray containing 10,000 gene clone immobilized cDNA fragments. Fluorescence intensities of Cy3 and Cy5 targets were measured and scanned separately using a GenePix 400B Array Scanner (Axon Instruments). Data analysis was done using GenePix Pro 3.0.5.56 (Axon Instruments) and GeneSpring GX 7.3.1 software (Agilent). The average-linkage distance was used to assess the similarity between two
groups of gene expression profiles as described below. The difference in distance between two groups of sample expression profiles to a third was assessed by comparing the corresponding average linkage distances [the mean of all pairwise distances (linkages) between members of the two groups concerned]. The error of such a comparison was estimated by combining the SE (the SD of pairwise linkages divided by the square root of the number of linkages) of the average-linkage distances involved. Classical multidimensional scaling (MDS) was carried out using the standard function of the R program to provide a visual impression of how the various sample groups are related (24).

**Quantitative real-time reverse-transcriptase-PCR**

Real-time reverse transcriptase-PCR (RT-PCR) was done as previously described (23). Briefly, total RNA (1 μg) of each sample was reverse-transcribed in a 20-μL reaction using 0.5 μg oligo(dT) and 200 U Superscript II RT (Invitrogen). The primer sequences used for real-time RT-PCR are shown in Supplementary Table S1. The amplification was carried out in a total volume of 20 μL containing 0.5 μmol/L of each primer, 4 mmol/L MgCl₂, 2 μL LightCycler-FastStart DNA Master SYBR green I (Roche Molecular Systems), and 2 μL of 1:10 diluted cDNA. PCR reactions were prepared in duplicate and heated to 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 55°C for 5 seconds, and extension at 72°C for 20 seconds. Standard curves (cycle threshold values versus template concentration) were prepared for each target gene and for the endogenous reference (GAPDH) in each sample. Quantification of unknown samples was done using LightCycler Relative Quantification Software version 3.3 (Roche Molecular Systems).

**Western blot assay**

The extraction of proteins from cells and Western blot analysis were done as described (23). A sample (15 μL) was boiled at 95°C for 5 minutes and separated by 10% SDS-PAGE. The proteins were wet-transferred to Hybond-ECL nitrocellulose paper (Amersham). Primary antibodies were as follows: rabbit anti-human phospho-STAT3, rabbit anti-human STAT3, rabbit anti-human cleaved poly(ADP-ribose) polymerase (PARP), rabbit anti-human cleaved caspase 3, and mouse anti-human survivin (Cell Signaling Technology); rabbit anti-human p21 (Santa Cruz Biotechnology); mouse anti-human Bcl-2 and rabbit anti-human Bax (Upstate); and mouse anti-β-actin (Chemicon). The reactive protein bands were detected by the ECL detection system (Amersham).

**Enzyme-linked immunosorbent assay, immunohistochemistry staining, and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay**

The activities of caspase-3 was determined using an enzyme-linked immunosorbent assay kit (R&D Systems) and quantified at 490 nm (MRX, Dynatech Laboratories). Each individual sample was analyzed in triplicate. The protocol of immunofluorescence staining has been described previously (23). Briefly, an avidin-biotin complex method was used for the immunofluorescence staining in the spheroid cells. Each slide was treated with antibodies for CD44 (DAKO), ALDH1 (Abcam), and phospho-STAT3 (Cell Signaling Technology). Immunoreactive signals were detected with a mixture of biotinylated rabbit anti-mouse IgG and Fluoresave (Calbiochem). Positive cells were counted in six different fields by microscopy. Furthermore, apoptotic cells were identified by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method (In situ Cell Death Detection Kit, POD, Roche Boehringer Mannheim Corp.; ref. 23).

**In vitro cell invasion analysis and soft agar assay**

The 24-well plate Transwell system with a polycarbonate filter membrane (8 μm pore size; Corning) was used. Cell suspensions were seeded in the upper compartment of the Transwell chamber at a density of 1 × 10⁵ cells in 100 μL serum-free medium. The opposite surface of the filter membrane, which faced the lower chamber, was stained with Hoechst 33342 for 3 minutes, and migrating cells were visualized under an inverted microscope. For the soft agar assay, the bottom of each well (35 mm) of a 6-well culture dish was coated with a 2 mL agar mixture [DMEM with 10% (v/v) FCS and 0.6% (w/v) agar]. After the bottom layer solidified, 2 mL top agar/medium mixture (DMEM with 10% FCS and 0.3% agar) containing 2 × 10⁴ cells was added and incubated at 37°C for 4 weeks. The plates were stained with 0.5 mL 0.005% crystal violet, and the number of colonies was counted using a dissecting microscope.

**In vivo analysis of tumor growth and metastasis**

All procedures involving animals were in accordance with the institutional animal welfare guideline of Taipei Veterans General Hospital. HNSCC-CD44⁺ALDH1⁺ cells (2 × 10⁵) were injected into the neck region of 8-week-old nude mice (BALB/c strain; ref. 25) and then treated with daily i.p. injections of vehicle (10% ethanol) or cucurbitacin I (1 mg/kg JSI-124 in 10% ethanol) for a total of 5 days (25). In vivo green fluorescent protein (GFP) imaging was done using an illuminating device [LT-9500 Illumatool TLS equipped with excitation illuminating source (470 nm) and filter plate (515 nm); ref. 23]. Tumor size was measured using calipers, and the volume was calculated according to the formula: (length × width²)/2 and subsequently analyzed using Image Pro-plus software (23).

**Statistical analysis**

The results are reported as mean ± SD. Statistical analysis was done using Student's t-test or a one-way or two-way ANOVA test followed by Turkey's test, as appropriate. Survival was estimated by the Kaplan-Meier
method and compared by the log-rank test. $P < 0.05$ was considered statistically significant.

**Results**

**Poor overall survival rate of patients with HNSCC is positively associated with CD44, ALDH1, and p-STAT3 expression**

To investigate whether there is a positive correlation among CD44, ALDH1, and p-STAT3 in head and neck cancers, we studied the levels of these proteins by immunohistochemical staining of a panel of specimens from 111 HNSCC patients. The immunohistochemistry results showed that increased expression of ALDH1 was positively correlated with the advanced stages and medium to poor differentiation of HNSCC (Fig. 1A). There was a significantly high correlation between tumor grade and ALDH1/CD44/p-STAT3 (relative coefficient $R = 0.87$ and 0.92 in low-grade and high-grade tumors, respectively; Fig. 1A). To determine the prognostic significance of CD44, ALDH1, and p-STAT3 expression in patients with HNSCC, we carried out Kaplan-Meier survival analysis. First, we found a significant difference for the 5-year survival prognosis between high-grade and low-grade HNSCC patients (Fig. 1B; $P < 0.001$). Second, the results of Kaplan-Meier survival analysis showed that the ALDH1-positive cases were associated with a considerably worse overall survival rate compared with ALDH1-negative ones (Fig. 1B; $P < 0.001$). Third, patients with lower CD44 expression had a better survival prognosis compared with the CD44-highly expressing patients (Fig. 1B; $P = 0.002$). Fourth, p-STAT3-positive patients had a worse survival prognosis (Fig. 1B; $P < 0.001$). Besides, there was a significantly high correlation between tumor grade and ALDH1+/CD44+/p-STAT3+ (relative coefficient $R = 0.87$ and 0.92 in low-grade and high-grade tumors, respectively). Taken together, these results suggest that elevated expression of CD44, ALDH1, and p-STAT3 were strongly associated with advanced grade of HNSCC and worse prognosis. In addition, patients positive for all three had the worst survival rate compared with other HNSCC patients (Fig. 1C; CD44+ALDH1+ p-STAT3+ versus other groups). Overall, these data indicate that expression of CD44, ALDH1, and p-STAT3 in HNSCC patients could be a critical factor in predicting disease progression and clinical outcomes.

**Isolation and characterization of CD44+ALDH1+ cells from HNSCC tissues**

Using the Aldefluor assay and FACS analysis, we isolated ALDH1+, ALDH1-, CD44+, and CD44- cells from tissue samples of seven HNSCC patients as previously described (ref. 8; Fig. 2A, left, and Supplementary Table S2). It has been reported that CSC-like cells can be cultured in suspension to generate floating spheroid-like bodies in serum-free medium with bFGF and EGF. In DF-12 serum-free medium with bFGF and EGF, the ability to form spheroid-like bodies and the proliferation rate in the ALDH1+-lineage (ALDH1+ and CD44+) cells were both significantly higher than in parental, ALDH1-, and CD44- cells (patients 1 to 3; $P < 0.001$; Fig. 2A, right; Supplementary Table S2). To evaluate the enhancement of tumorigenicity of HNSCC-ALDH1+ cells, we employed Matrigel/Transwell invasion and soft agar colony formation assays. Compared with ALDH1+ or CD44+ALDH1+, CD44+ALDH1- cells derived from HNSCC patients 1, 2, and 3 showed higher invasion activity, as assessed by the Matrigel/Transwell invasion assay ($P < 0.001$; Fig. 2B, left). Similarly, the colony formation ability of ALDH1+ and CD44+ALDH1+ cells from HNSCC patients was enhanced when compared with the ALDH1+ or CD44+ALDH1+ of the same patient ($P < 0.001$; Supplementary Fig. S2A). We further found that the stemness genes (Oct-4A and Nanog), epithelium-mesenchymal transition (EMT) transcriptional factors (Snail and Twist), and the drug-resistant genes (MDR-1) were upregulated in both ALDH1+ and CD44+ALDH1+ cells using real-time RT-PCR (Fig. 2B, right; Supplementary Fig. S2B). To further determine the effect of radiation on tumor growth rate, we used ionizing radiation doses from 0 to 10 Gy to treat the tumors in these five groups. As shown in Fig. 2C, after ionizing radiation treatment, both the survival rate and number of ALDH1+ and CD44+ALDH1+ cells were significantly higher than in ALDH1- and CD44+ALDH1+ (P < 0.001). Because several studies have shown that STAT3 activation is related to the malignancy of HNSCCs, the activation status of STAT3 in ALDH1+ and ALDH1- HNSCC cells was detected. As shown in Fig. 2D, the levels of activated STAT3 (p-STAT3-Tyr705) in CD44+ALDH1+ cells were significantly higher than parental cells both in serum-free and serum-containing medium. These findings suggest the p-STAT3 may play some roles in regulating the property of HNSCC-CD44+ALDH1+.

**Cucurbitacin I inhibits the proliferation, tumor malignancy, and stemness signatures of CD44+ALDH1+ cells**

Cucurbitacin I, a specific STAT3 inhibitor, has been recently suggested to suppress tumor growth. It remains undetermined, however, whether cucurbitacin I can inhibit the CSC properties of HNSCC-CD44+ALDH1+ cells. The viability of HNSCC-CD44+ALDH1+ cells determined by MTT assay significantly decreased with increasing concentrations of cucurbitacin I ($P < 0.05$; Fig. 3A). Treatment with cucurbitacin I also significantly blocked the colony formation capability of HNSCC-CD44+ALDH1+ cells ($P < 0.05$; Fig. 3B). To explore molecules governing stemness and tumorigenicity in HNSCC-CD44+ALDH1+ cells treated with cucurbitacin I, we examined their transcriptome profile using gene expression microarray analysis (Fig. 3C; Supplementary Tables S3 and S4). MDS and principle component analysis (PCA) further showed that HNSCC-CD44+ALDH1+ cells were more similar to high-grade tissues of HNSCC than low-grade HNSCC or normal oral tissues (Fig. 3D). In contrast, the MDS results showed that the expression patterns of ALDH1+,
Figure 1. Correlation of CD44, ALDH1, and p-STAT3 expression to the clinical grading and survival rate of HNSCC patients. A, left, representative results of immunohistochemical staining for ALDH1 (top), CD44 (middle), and p-STAT3 (bottom) in 111 HNSCC patients at different grades (left, low grade; right, high grade). Right, significantly high correlation between tumor grade and ALDH1, CD44, and pSTAT3 (relative coefficient $R = 0.87$ and $0.92$ in low-grade and high-grade tumor, respectively). B, Kaplan-Meier analysis of overall survival in 111 HNSCC patients according to clinical histology grading (top left; $P < 0.001$), single ALDH1 expression (bottom left; *, $P < 0.001$), single CD44 expression (top right; **, $P = 0.002$), single p-STAT3 expression (bottom right; *, $P < 0.001$), and combined expression of CD44$^+$ALDH1$^+$p-STAT3$^+$ (C; ***, $P < 0.001$). Inset, CD44$^-$ALDH1$^-$p-STAT3$^-$ cells (C) used as reference group for comparison.
Figure 2. Isolation and characterization of CD44+ALDH1+ cells from HNSCC tissues. A, 6.15% CD44+ALDH1+ cells were identified from HNSCC tissues via FACScan. DEAB, an inhibitor of ALDH, was used as negative control (left). The ability to form spheroid-like bodies in various groups in serum-free medium with bFGF and EGF was evaluated (right). Bar, 100 μm. B, invasion ability was detected in the different ALDH1+ and ALDH1− groups (left). *, P < 0.001, ALDH1+ versus parental and ALDH1−; #, P < 0.001, CD44+ALDH1+ versus ALDH1+. Q-RT-PCR results to quantify the amounts of transcripts of indicated genes (right). C, to determine the radiation effect on the tumor growth rate, ionizing radiation doses of 0 to 10 Gy were used to treat ALDH1+ and ALDH1− cells. D, protein levels of activated STAT3 (p-STAT3-Tyr705) and total STAT3 from ALDH1+ and parental cells in serum-free (top) and serum-containing (bottom) medium were determined by Western blot.
Figure 3. Microarray analysis reveals key cucurbitacin I–regulated transcriptomes. A, HNSCC-CD44+ALDH1+ cells from patients 1 to 3 were plated in 24-well plates and incubated for 48 hours with various concentrations of cucurbitacin I. At the end of treatment, cell viability was determined by MTT assay. B, treatment with cucurbitacin I in HNSCC-CD44+ALDH1+ cells impeded the capability of colony formation as evaluated by soft agar assay. *, P < 0.001. C, gene expression microarray analysis (gene tree) of the 987 genes that were differentially expressed in cucurbitacin I–treated (cu-) HNSCC-CD44+ALDH1+ cells as compared with control cells as shown by a hierarchy heat map. The time-dependent changes in expression of the...
Cucurbitacin I-treated CD44+ALDH1+ cells, and low-grade HNSCC were more close to the normal oral tissues. Notably, microarray analysis showed that the expression of 987 probe sets was significantly altered in the cucurbitacin I-treated group compared with the control group when compiled with the hierarchical clustering method (Fig. 3C). More importantly, the treatment of cucurbitacin I in HNSCC-CD44+ALDH1+ cells resulted in an extension of the average linkage distances among CD44+ALDH1+, ALDH1+, and high-grade HNSCC (P < 0.05; Fig. 3D).

**Cucurbitacin I promotes differentiation and induces apoptosis by blocking STAT3 signaling in HNSCC**

To examine whether CSC-like properties are suppressed via STAT3 inhibition, HNSCC-CD44+ALDH1+ cells were incubated with 50, 100, or 150 nmol/L cucurbitacin I for 24 hours. Treatment with cucurbitacin I significantly interfered with the formation of spheroid-like bodies (Fig. 4A). Importantly, the Aldefluor assay and FACS analysis showed that the quantities of ALDH1 and CD44 were dramatically decreased in cucurbitacin I–treated HNSCC-ALDH1+CD44+ cells (Fig. 4B; P < 0.001). Western blotting showed that cucurbitacin I induced a dose-dependent decrease of p-STAT3 in HNSCC-CD44+ALDH1+ cells (Fig. 4C). To elucidate the effects of cucurbitacin I on STAT3 downstream genes and cell survival–related genes, the expressions of survivin, Bcl-2, Bcl-xL, and Bax were examined by Western blot. As shown in Fig. 4C, Bcl-2, Bcl-xL, and survivin expression were downregulated, whereas Bax production was enhanced with cucurbitacin I treatment. The amount of cleaved PARP and caspase 3 was obviously increased (Fig. 4C). Consistent with this finding, TUNEL staining also revealed that apoptotic signals were positively correlated with cucurbitacin I concentration (Fig. 4D). We further found that cucurbitacin I suppressed Stat3 and Janus-activated kinase 2 (JAK2) activation without influencing Src phosphorylation (Fig. 4C). These data suggest that the STAT3 pathway maintains the stemness of ALDH1+CD44+ cells and the inhibition of STAT3 activation further promotes apoptosis in HNSCC-CSC.

**Cucurbitacin I improves sensitivity to radiotherapy in HNSCC-CD44+ALDH1+ cells**

To further investigate the biological roles of STAT3 in tumorigenicity of HNSCC-CD44+ALDH1+ cells under radiation treatment, we applied varying ionizing radiation doses from 0 to 10 Gy to vehicle- or cucurbitacin I–treated HNSCC-CD44+ALDH1+ cells. As shown in Fig. 5A, the survival rate of vehicle-treated HNSCC-CD44+ALDH1+ cells was significantly higher than that of cells treated with 100 nmol/L cucurbitacin I (P < 0.01). To further explore the mechanism involved in the cucurbitacin I–mediated radiosensitizing effect against HNSCC cells, the 100 or 150 nmol/L cucurbitacin I–treated HNSCC parental cells or HNSCC-CD44+ALDH1+ cells were exposed to 4 Gy ionizing radiation. The capabilities of colony formation (Fig. 5B), invasion (Fig. 5C, left), and sphere formation (Fig. 5C, right) were dramatically attenuated by cucurbitacin I and by ionizing radiation. In addition, the combination treatment showed a synergistic effect in abrogating these HNSCC cell capabilities. Cell viability assays showed that the cytotoxic effect of 4 Gy ionizing radiation on HNSCC-CD44+ALDH1+ cells was significantly increased with the addition of 100 nmol/L cucurbitacin I (P < 0.01; Fig. 5D, left). Meanwhile, the activity of caspase 3 was concomitantly increased in HNSCC-CD44+ALDH1+ cells treated with cucurbitacin I plus ionizing radiation compared with ionizing radiation treatment alone (Fig. 5D, right). These data indicate that the effectiveness of radiation treatment on HNSCC-CD44+ALDH1+ cells can be improved with cucurbitacin I, an effect mediated by the suppression of STAT3 signaling.

**Cucurbitacin I presents the synergistic effects with ionizing radiation to inhibit tumorigenicity and distant-metastatic ability in HNSCC-ALDH1+-transplanted immunocompromised mice**

We further investigated the role of the STAT3 signaling and the effects of cucurbitacin I in HNSCC-CD44+ALDH1+ cells in vivo. HNSCC-ALDH1+/- and HNSCC-parental cells were transfected with a lentiviral vector containing GFP (22–24). We first injected 2 × 10^5 CD44+ALDH1+/-GFP, 2 × 10^5 ALDH1+/-GFP, and 2 × 10^5 HNSCC parental cells-GFP cells into the neck region of nude mice that received different treatment protocols (Fig. 6). First, we found that the subset of nude mice receiving ALDH1+/-GFP cells formed no tumors in the neck region within 10 weeks of xenotransplantation (data not shown). CD44+ALDH1+/-GFP cells presented the strongest ability to form tumors in transplanted mice (Fig. 6A). Cucurbitacin I (1 mg/kg, i.p. for 5 days) effectively suppressed the proliferation of CD44+ALDH1+/-GFP cells in the transplanted mice (Fig. 6A). Notably, cucurbitacin I showed a synergistic effect with ionizing radiation in CD44+ALDH1+/-GFP–transplanted mice compared with mice receiving the same cells but exposed to ionizing radiation alone (P < 0.05; Fig. 6A). Furthermore, our in vivo data show that the subset of CD44+ALDH1+ cells, but not the ALDH1+–lineage cells or HNSCC-parental cells group, exhibited significant capabilities of invasion and distant metastasis to the lungs (Fig. 6B). Importantly, cucurbitacin I treatment in CD44+ALDH1+/-GFP–transplanted mice effectively reduced the number of lung metastases and tumor size in vivo (Fig. 6B, Supplementary Fig. S3). Further combination with 4 Gy ionizing radiation showed a significant diminution in the multiple nodules of tumor formation and the reduction of tumor volume in the CD44+ALDH1+ group, suggesting that cucurbitacin I plus ionizing radiation synergistically blocked the metastatic ability of CD44+ALDH1+ cells (Fig. 6B, Supplementary Fig. S3). Furthermore, immunohistochemistry showed that the expression levels of p-STAT3 in the neck region tumors of HNSCC CD44+ALDH1+–injected nude mice were highly
expressed in comparison with the other groups ($P < 0.05$; Fig. 6C). Our result further showed that p-STAT3 immunohistochemistry levels were significantly decreased in CD44$^{+}$ALDH1$^{-}$xenotransplanted graft after the treatment of cucurbitacin I ($P < 0.001$; Fig. 6C). Moreover, CD44$^{+}$ALDH1$^{-}$-GFP-transplanted mice treated with the combination of 4 Gy ionizing radiation and cucurbitacin I had a mean survival rate that was significantly prolonged compared with the control group and CD44$^{+}$ALDH1$^{-}$-GFP-transplanted mice that received other
Figure 5. Cucurbitacin I suppresses CSC-like properties of HNSCC-CD44+ALDH1+ cells by inhibiting STAT3 signaling and improves the sensitivity to radiotherapy. A, to determine the effect of radiation on tumor growth rate, ionizing radiation (IR) doses from 0 to 10 Gy were used to treat HNSCC-CD44+ALDH1+ cells in combination with vehicle or cucurbitacin I (Cu). Colony formation (B) and the invasion abilities (C) of parental and HNSCC-CD44+ALDH1+ cells were examined after treatment with either cucurbitacin I or 4 Gy ionizing radiation or both (C, left). Treatment with cucurbitacin I combined with irradiation in parental and HNSCC-CD44+ALDH1+ cells impeded the capability to form spheroid-like bodies (C, right). *, P < 0.001. D, cell viability (MTT assay) and caspase 3 activity were determined in HNSCC-CD44+ALDH1+ cells after treatment with 4 Gy ionizing radiation in the presence or absence of cucurbitacin I. Data shown are the mean ± SD of three independent experiments.
Figure 6. Evaluation of in vivo tumorigenicity of HNSCC-CD44+ALDH1+ cells and survival time in a xenotransplanted animal model. A, a total of 2 × 10^5 HNSCC-CD44+ALDH1+ and HNSCC-parental cells were injected s.c. into the necks of nude mice. Six mice in each group (n = 6 in each group; total 36 mice) received daily i.p. injections of vehicle (10% ethanol) or drug [1 mg/kg cucurbitacin I (Cucur/Cu) in 10% ethanol]. After 4 weeks, in vivo GFP imaging revealed that transplanted HNSCC-CD44+ALDH1+-GFP cells grew solid tumors in the injection site. Tumor volumes in HNSCC-CD44+ALDH1+-transplanted mice treated with cucurbitacin I (1 mg/kg, i.p. for 5 days) with ionizing radiation (4 Gy) were significantly lower than in those receiving ionizing radiation or cucurbitacin I only (P < 0.01). B, the number of metastatic foci (top left, fluorescence spots; arrows) and total volume of tumors in the lungs of mice were analyzed by macroscopic and histologic examination (bottom left and middle; arrow, neovascularity and thrombosis). Cucurbitacin I only or combined with 4 Gy ionizing radiation effectively reduced the number of lung metastases and tumor size in CD44+ALDH1+–transplanted mice (*, P < 0.01; n = 6 in each group; total 36 mice). C, immunohistochemistry for p-STAT3 indicated that cucurbacitin I effectively suppressed the level of p-STAT3 in CD44+ALDH1+– and parental cell–xenotransplanted immunocompromised mice. Bar, 20 μm. Data shown are the mean ± SD of three experiments. D, Kaplan–Meier survival analysis further indicated that the mean survival rate for animals receiving HNSCC-CD44+ALDH1+ cells treated with cucurbitacin I combined with ionizing radiation was significantly prolonged compared with those receiving ionizing radiation or drug along (each group n = 10 mice).
treatments (P < 0.05; Fig. 6D). Overall, this in vitro study shows that the effectiveness of ionizing radiation in mice bearing HNSCC-CD44+ALDH1+ tumors can be significantly improved with the addition of cucurbitacin I treatment.

**Discussion**

STAT3, a transcription factor regulated by various cytokines and growth factors, especially interleukin-6 (IL-6) and EGF, is aberrantly activated in numerous cancer types, including HNSCC. Targeting STAT3 signaling by introducing dominant-negative constructs, decoy oligonucleotides, or antisense oligonucleotides to HNSCC cells significantly inhibits growth and induces apoptosis (26). Because persistent activation of STAT3 promotes tumor cell proliferation and survival, further contributing to tumor progression and migration, abrogation of STAT3 signaling is emerging as a potential cancer therapy strategy. Herein we found that levels of CD44, ALDH1, and p-STAT3 were greater in higher-grade HNSCC tissues than in lower-grade samples (Fig. 1). The immunohistochemical analysis showed that triple positivity for CD44, ALDH1, and p-STAT3 indicated a worse prognosis for HNSCC patients (Fig. 1). Consistent with this observation, we found that the phosphorylation levels of STAT3 in CD44+ALDH1+ cells from seven HNSCC patients were significantly higher than those in the CD44−, ALDH1−, and parental cells (Fig. 2). Moreover, consistent with our previous findings (27), CD44+ALDH1+ cells isolated from HNSCC patients shared characteristics of CSC and showed greater radioreistance compared with HNSCC cells of the CD44− or ALDH1− lineage (Fig. 2). Cucurbitacins, triterpenoid derivatives, are strong inhibitors of the JAK/STAT pathway and exhibit biopharmacologic activities in anticancer effects. Our data showed that 150 nmol/L cucurbitacin I could effectively block STAT3 signaling and further induce apoptosis in HNSCC-CD44+ALDH1+ (Fig. 3). Notably, 100 nmol/L cucurbitacin I blocked formation of spheroid-like bodies and facilitated CD44+ALDH1+ differentiation into CD44+ALDH1− cells (Fig. 3). Moreover, 100 nmol/L cucurbitacin I enhanced radiosensitivity in CD44+ALDH1+ cells (Fig. 3). Notably, the result of immunohistochemistry showed that cucurbitacin I can effectively inhibit the expression of p-STAT3 in CD44+ALDH1−→xenotransplanted mice (Fig. 6C). Finally, in vivo xenotransplant analysis indicated that the mean survival rate of mice bearing HNSCC-CD44+ALDH1+ cells and treated with ionizing radiation could be significantly improved by adding cucurbitacin treatment (Fig. 6). To our knowledge, this is the first study to show that the STAT3 axis plays an important role in maintaining CSC-like properties and that targeting STAT3 with cucurbitacin I significantly suppresses tumorigenicity and radioresistance in HNSCC-associated CSCs.

Radiotherapy is the conventional treatment for HNSCC. Radioresistance is one of the major causes of tumor recurrence and metastasis in HNSCC. To improve the therapeutic outcome of malignant HNSCC and further specifically target HNSCC-associated CSCs, novel therapeutic agents and radiosensitizers are urgently needed. STAT3 is an important component of several signaling pathways, such as that of EGF, which activates genes required for DNA repair and cell survival after radiation treatment. Recent reports have suggested that a combination of STAT3 inhibitor and radiotherapy effectively attenuated tumor cell growth. Introducing STAT3 siRNA significantly enhanced radiosensitivity in human laryngeal squamous cell carcinoma cells (28) and in human squamous cell carcinoma cells with EGFR overexpression (29). Chen et al. further showed that the IL-6/STAT3 pathway is responsible for the resistance to EGFR inhibitor and irradiation in pharyngeal cancer (29). Clinical specimens further assessed by immunohistochemistry have shown that higher levels of IL-6, IL-6 receptor, and p-STAT3 are correlated with lower response rates to treatments and shorter survival (29). Moreover, panitumumab, a monoclonal antibody against EGFR, can significantly augment the radiosensitivity of HNSCC and non-small cell lung cancer in vitro and in vivo (30). Therefore, the inhibition of IL-6/STAT3 or p-STAT3 may be an important therapy when combined with conventional radiotherapy. In this study, our data suggest that cucurbitacin I (JSI-124) significantly increased ionizing radiation–induced apoptosis and suppressed the radiosensitivity in HNSCC-CSC, in part through the inactivation of p-STAT3 and downstream survivin and Bcl-2 expression (Fig. 3). According to clinical follow up, our data also indicated that the combined high expression of p-STAT3, CD44, and ALDH1 in HNSCC was highly correlated with the clinical radioresistant history (data not shown). Importantly, the in vivo tumorigenic and metastatic capabilities of HNSCC-CD44+ALDH1+ cells treated with 4 Gy ionizing radiation combined with 100 nmol/L cucurbitacin I were significantly decreased compared with those of untreated CD44+ALDH1+ and only ionizing radiation–treated CD44+ALDH1+ cells (Fig. 5). Indeed, these findings indicate the antiproliferative, proapoptotic, and radiosensitizing effect of cucurbitacin I on CD44+ALDH1+ cells and could potentially be used to improve the clinical treatment of HNSCC-CSC as well as advanced-stage HNSCC patients. In addition, a recent study also suggests that cucurbitacin could potentially attenuate the proinflammatory effect of IL-6 through downregulation of IL-6/STAT3 signaling (31, 32). Furthermore, recent evidence has reported the association between STAT3 and EMT. Exogenous addition of EGF induced migratory phenotype, enhanced IL-6 production, and upregulated the level of N-cadherin and vimentin in the epithelial ovarian cancer lines OVCA 433 and SKOV3. These enhancements were abrogated as the STAT3 pathway was blocked by neutralizing IL-6R antibody and AG490 (33). Likewise, ectopic IL-6 expression in the breast adenocarcinoma cell line MCF-7 promoted EMT phenotype, including impaired E-cadherin expression and induction of vimentin, N-cadherin, Snail, and Twist (34).
In this study, our in vivo data showed that CD44+ ALDH1+–lineage cells exhibited significant capabilities of invasion and distant metastasis to the lungs, which was not found in CD44− or ALDH1− HNSCC cells (Fig. 6). The results of microarray analysis and quantitative RT-PCR further showed that the treatment of cucurbitacin I inhibited the constitutive mRNA expressions of Snail and Twist in treated HNSCC-CD44+ALDH1+ cells (Supplementary Fig. S4). Notably, our data showed that cucurbitacin I presents the potential to suppress the metastatic ability to lung organ in HNSCC-CD44+ALDH1+–transplanted nude mice (Fig. 6). These findings suggest that STAT3 pathway may be involved in the molecular signaling of EMT and distant metastasis in head and neck cancer in vivo. Moreover, some reports have shown the synergistic effect of cucurbitacins with known chemotherapeutic agents, such as doxorubicin (35). Although the molecular mechanism of HNSCC-CSC or CD44+ALDH1+ is still unclear, there is a need to further investigate targetting IL-6/JAK/STAT3 signaling as well as other paracrine or autocrine factors and radiochemoattractants involved in the tumor microenvironment that biologically impact HNSCC progression.

In summary, our data indicate that the STAT3 signaling axis may contribute to the CSC-like properties and radioresistance of HNSCC-CD44+ALDH1+ cells. We identified cucurbitacin I (JSI-124), a selective JAK/STAT3 signaling pathway inhibitor, as a potent antitumor agent in HNSCC-CSC in vivo and in vitro. Notably, cucurbitacin I enhanced the inhibition of cancer stem-like property, blockage of invasion ability, and induction of apoptosis by ionizing radiation in HNSCC-CSC. The potential of using triple expression of CD44, ALDH1, and p-STAT3 as a marker of radioresistance in HNSCC or other solid tumors should be verified in future clinical trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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