Targeting Signal Transducer and Activator of Transcription 3 Pathway by Cucurbitacin I Diminishes Self-Renewing and Radiochemoresistant Abilities in Thyroid Cancer-Derived CD133\(^+\) Cells

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ABSTRACT

Anaplastic thyroid cancer (ATC) is a lethal solid tumor with poor prognosis because of its invasiveness and its resistance to current therapies. Recently, ATC-CD133\(^+\) cells were found to have cancer stem cell (CSC) properties and were suggested to be important contributors to tumorigenesis and cancer metastasis. However, the molecular pathways and therapeutic targets in thyroid cancer-related CSCs remain undetermined. In this study, ATC-CD133\(^+\) cells were isolated and found to have increased tumorigenesis, radioresistance, and higher expression of both embryonic stem cell-related and drug resistance-related genes compared with ATC-CD133\(^-\) cells. Microarray bioinformatics analysis suggested that the signal transducer and activator of transcription 3 (STAT3) pathway could be important in regulating the stemness signature in ATC-CD133\(^+\) cells; therefore, the effect of the potent STAT3 inhibitor cucurbitacin I in ATC-CD133\(^+\) cells was evaluated in this study. Treatment of ATC-CD133\(^+\) cells with cucurbitacin I diminished their CSC-like abilities, inhibited their stemness gene signature, and facilitated their differentiation into ATC-CD133\(^-\) cells. Of note, treatment of ATC-CD133\(^+\) cells with cucurbitacin I up-regulated the expression of thyroid-specific genes and significantly enhanced radioiodine uptake. Furthermore, cucurbitacin I treatment increased the sensitivity of ATC-CD133\(^+\) cells to radiation and chemotherapeutic drugs through apoptosis. Finally, xenotransplantation experiments revealed that cucurbitacin I plus radiochemotherapy significantly suppressed tumorigenesis and improved survival in immunocompromised mice into which ATC-CD133\(^+\) cells were transplanted. In summary, these results show that the STAT3 pathway plays a key role in mediating CSC properties in ATC-CD133\(^+\) cells. Targeting STAT3 with cucurbitacin I in ATC may provide a new approach for therapeutic treatment in the future.

Introduction

Thyroid cancer is a common endocrine tumor. Approximately 85 to 90% of patients with thyroid cancer are diagnosed with papillary thyroid cancer (PTC), which originates from thyroid follicular cells (Sipos and Mazzafferri, 2010). Patients with PTC are expected to survive for 10 years or more after diagnosis (Sipos and Mazzafferri, 2010). However,
patients with anaplastic thyroid cancer (ATC), an undifferentiated thyroid cancer, have a poor prognosis with a median survival of less than 6 months after traditional treatments such as surgery, radiotherapy, and chemotherapy (Sugino et al., 2002; Sipos and Mazzaferrri, 2010). Cancer stem cells (CSCs), a crucial subset within the bulk of the tumor, exhibit self-renewal and differentiation capabilities. Some studies have demonstrated that CSCs exist in many cancer types and are responsible for tumor development, relapse, and metastasis (Jordan et al., 2006). Zito et al. (2008) identified CD133+ cells from four human ATC cell lines that presented stem cell-like features in vitro. In addition, Friedman et al. (2009) evaluated the tumor initiation potential of CD133+ ATC cells in an animal model. Together, their results suggest that the CD133 subsets within ATC may account for tumor progression, resistance to current modes of chemotherapy, and the aggressiveness of ATC.

Signal transducer and activator of transcription 3 (STAT3), a transcription factor that responds to cytokine and growth factor signaling, regulates a variety of cellular functions via its downstream target genes such as p21, c-Myc, and cyclin D1 (cell cycle progression genes); matrix metalloproteinase 9 (MMP-9) (mediating cellular invasion); and vascular endothelial growth factor (regulating angiogenesis) (Yu and Jove, 2004). STAT3 was found to be constitutively activated in numerous cancer types (Bowman et al., 2000; Broombberg, 2001, 2002; Epling-Burnette et al., 2001), and it was also 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 (Bromberg et al., 1999; Niu et al., 2005). Previous reports have shown that STAT3 can be directly activated by rearranged in transformation/papillary thyroid carcinoma (RET/PTC) tyrosine kinase, the most frequently identified genetic rearrangement in PTC (Hwang et al., 2003). Several proteins such as phospholipase D and tumor suppressor LKB1 have been suggested to be modulators of RET/PTC-induced STAT3 activation (Kim et al., 2007, 2008). Another study demonstrated that p53 mutations in ATC play an important role in tumor progression via the STAT3 pathway (Kim et al., 2009). Abolishment of STAT3 phosphorylation by exogenous expression of suppressors of cytokine signaling or administration of a RET/PTC kinase inhibitor inhibits the growth of thyroid carcinoma cells, suggesting that targeting the STAT3 pathway could be useful in thyroid cancer therapy (Kim et al., 2006; Francipane et al., 2009). Although recent studies have shown that STAT3 activation is strongly associated with thyroid tumorigenesis, the mechanisms of STAT3 signaling pathways need to be investigated further to identify possible therapeutic targets for thyroid CSCs.

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 (Lee et al., 2003). Cucurbitacins have been used as folk medicine since ancient times and are known for their wide range of biological activities (Chalfont St. Giles, Buckinghamshire, UK) cDNA probes for control (Gibco-BRL) to generate Cy3- and Cy5-labeled (GE Healthcare, Roche Molecular Systems, Inc.).

Materials and Methods

Isolation of CD133+ Cells from Thyroid Cancer Patients and Cell Lines. This research followed the tenets of the Declaration of Helsinki, and all samples were obtained after patients provided informed consent. Three cell lines (BHT-101, CAL-62, and 8505c) and cells dissociated from a sample from a patient with ATC were labeled with 1 μl of CD133/micromagnetic beads per 1 million cells using a CD133+ cell isolation kit (Miltenyi Biotech, Auburn, CA). CD133+ cells were cultured in medium consisting of serum-free DMEM/F12 (Invitrogen, Carlsbad, CA) with N2 supplement (R&D Systems Inc., Minneapolis, MN), 10 ng/ml human recombinant bFGF (R&D Systems Inc.), and 10 ng/ml EGF (R&D Systems Inc.) (Chiou et al., 2008).

Irradiation and Clonogenic Assay. Ionizing radiation (IR) was delivered by a cobalt unit (Theratronic International, Inc., Ottawa, ON, Canada) at a dose rate of 1.1 Gy/min (source-to-surface distance = 57.5 cm). For a 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 min with a solution containing crystal violet and methanol. Cell survival was determined by a colony formation assay. The plating efficiency (PE) and survival fraction (SF) were calculated as follows: PE = (colony number/number of inoculated cells) × 100%. SF = colonies counted/(cells seeded × (PE/100)).

Quantitative Real-Time RT-PCR. Real-time RT-PCR was performed as described previously (Kao et al., 2009). In brief, total RNA (1 μg) from each sample was reverse-transcribed in a 20-μl reaction using 0.5 μg of oligo(dT) and 200 U of SuperScript II RT (Invitrogen) (Pierson et al., 2008). The primer sequences used for real-time RT-PCR are shown in Table 1. DNA amplification was performed in a total volume of 20 μl of 1:10 diluted cDNA. Duplicate PCRs were heated to 95°C for 10 min, followed by 40 cycles of the following: denaturation at 95°C for 10 s, annealing at 55°C for 5 s, and extension at 72°C for 20 s. Standard curves of cycle threshold values versus template concentrations were prepared for each target gene and for the endogenous reference (glyceraldehyde-3-phosphate dehydrogenase) in each sample. Unknown samples were quantitated using LightCycler Relative Quantification Software (version 3.3; Roche Molecular Systems, Inc.).

Microarray Analysis and Bioinformatics. Total RNA was extracted from cells using TRIzol reagent (Invitrogen) and the RNaseasy purification kit (Qiagen, Valencia, CA). Total RNA was reverse-transcribed with SuperScript II RNase H-reverse transcriptase (Gibco-BRL) to generate Cy3- and Cy5-labeled (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) cDNA probes for control and treated samples, respectively. The labeled probes were hybridized to a cDNA microarray containing immobilized cDNA fragments corresponding to 10,000 gene clones. Fluorescence intensities of Cy3 and Cy5 targets were measured and scanned separately using a GenePix 4000 B Array Scanner (Molecular Devices, Sunnyvale, CA). Data analysis was performed using GenePix Pro 3.0.5.56 (Molecular
proteins were transferred to Hybond-ECL nitrocellulose paper (GE Healthcare). Fifteen microliters of sample were boiled at 95°C for 5 min and blot analysis were performed as described previously (Kao et al., 2009). The reactive protein bands were detected with the ECL detection system (GE Healthcare).

**Western Blot Assay.** Protein extraction from cells and Western blot analysis were performed as described previously (Furuya et al., 2004). In brief, cells grown in six-well plates were incubated with 1 ml of Hanks’ balanced salt solution containing 0.2 µCi of carrier-free Na2125I (PerkinElmer Life Sciences, Waltham, MA) and 10 µM NaI with or without 300 µM NaClO4, a sodium/iodide symporter (NIS) inhibitor.

An iodide uptake assay was performed as described previously (Furuya et al., 2004). After incubation for 1 h at 37°C, cells were washed twice with ice-cold Hanks’ balanced salt solution and detached with trypsin and detached with trypsin and incubated at 37°C for 4 weeks. The plates were stained with 0.5 ml of 0.005% crystal violet, and the number of colonies was counted using a dissecting microscope.

**Statistical Analysis.** The Statistical Package of Social Sciences software (SPSS, Inc., Chicago, IL) was used for statistical analysis. An independent Student’s t test was used to compare the continuous variables between groups. The Kaplan-Meier procedure was used to calculate survival probability estimates. A log-rank test was used to compare the cumulative survival durations in different patient groups. The statistical significance level was set at 0.05 for all tests.

**Results**

**Isolation and Characterization of ATC-Derived CD133+ Cells.** Recent studies have shown that the expression of CD133 in thyroid cancer results in high tumorigenicity and resistance to conventional therapy (Zito et al., 2008; Friedman et al., 2009). To look for additional CSC-like characteristics in CD133+ cells from ATC cells and tissues, we isolated...
CD133+ cells (Fig. 1A, left) from tissue sample of 17 patients with ATC using the magnetic bead method. ATC-CD133+ cells formed floating spheroid-like bodies in DF-12 serum-free medium with bFGF and EGF (Fig. 1A, right). Quantitative real-time RT-PCR results showed that the transcript levels of “stemness” genes (Oct4, Sox2, and Nanog) and drug resistance genes (ABCG2, MDR1, and MRP) were higher in ATC-CD133+ cells than in ATC-CD133− cells (Fig. 1B). Furthermore, ATC-CD133+ cells displayed greater ability for sphere formation (Fig. 1C), foci formation (Fig. 1D), and invasive activity (Fig. 1E), as well as enhanced resistance to conventional chemotherapeutic drugs than ATC-CD133− cells of the same origin (**, *P < 0.05*) (Fig. 1, F–H). To evaluate the tumor-restoration capability of ATC-CD133+ cells, 3×10^3 and 3×10^4 ATC-CD133− cells from 12 patients, as well as 3×10^4 ATC-CD133+ cells from all 17 patients, developed into visible tumors 6 weeks after injection into xenotransplanted mice (Table 2). To further explore the key regulators governing stemness and tumorigenicity in ATC-CD133+ cells, we performed microarray studies to compare the transcriptional profile of CD133− and CD133+ cells (Fig. 2A). To gain additional insights into the functional consequences of differential gene expression patterns and to provide quantitative evidence, signature genes were submitted to the gene ontology database to identify statistically represented functional groups. The predominant processes up-regulated in ATC-CD133+ cells include transcription, activation of transmembrane receptor, protein tyrosine kinase activity, DNA repair, and stress response (Fig. 2A; Supplemental Table 1). In ad-

![Fig. 1. Isolation and characterization of CD133(+/−) cells from ATC tissues. A, left, with use of a magnetic bead method, CD133+ cells were sorted from tumor tissues and confirmed by flow cytometry. Red line, mock group; green line, isotype-stained group; purple line, CD133 antibody-stained group. Right, CD133+ and CD133− cells were cultured in bFGF- and EGF-supplemented DMEM serum-free medium. Scale bar, 100 μm. B, mRNA expression levels of Oct4, Sox2, Nanog, ABCG2, MDR1, and MRP in ATC-CD133+ and ATC-CD133− cells were determined by quantitative real-time RT-PCR. C to E, The spheroid-like body formation ability (C), tumor foci (soft agar colony) formation (D), and migration/invasion capabilities (E) of CD133+ cells from ATC tissues were significantly increased compared with those in CD133− cells (**, *p < 0.05*). F to H, A total of 2×10^4 cells were plated in a 24-well plate and treated with 20 μM cisplatin (F), 40 μM 5-FU (G), or 1.2 μM doxorubicin (H) for 24 h in 10% fetal calf serum/DMEM/F12 medium. The survival rate relative to that of nontreated groups (served as 100%) was determined by the MTT assay. P1, patient. *, **, *p < 0.05.”]
dition, using a literature-based network analysis of all MEDLINE records (title and abstract) and Cytoscape software to group the target-linkage genes, we found that STAT3 and p-STAT3 are potentially key factors regulating cancer-related biomolecular signatures and pathways in ATC-CD133 cells (Fig. 2B). Taken together, results from bioinformatics analysis and transcriptom profiling indicated that STAT3 and STAT3-related pathways play a critical role in the regulation of stemness and tumorigenicity of ATC-CD133 cells.

**Cucurbitacin I Suppresses Cell Proliferation and Attenuates CSC Properties in ATC-CD133 Cells.** Because several studies have demonstrated that STAT3 activation is associated with thyroid cancer, the activation status of STAT3 or p-STAT3 in ATC-CD133 and ATC-CD133 cells was further evaluated. Western blot results confirmed that the level of activated STAT3 was higher in CD133 cells than in CD133 cells or parental cells (Fig. 3A), in agreement with the microarray data. Cucurbitacin I is a selective JAK-STAT inhibitor that blocks the tyrosine phosphorylation of STAT3 and JAK2 but not that of other oncogenic or survival pathways such as the Akt, extracellular signal-regulated kinase, and JNK signaling pathways (Blaskovich et al., 2003). However, whether cucurbitacin I can attenuate the CSC properties of ATC-CD133 cells through inhibiting the activation of STAT3 remained unknown. To elucidate the possible role of cucurbitacin I in blocking ATC CSCs, we compared the different dosages (50, 100, and 150 nM) of cucurbitacin I in treated ATC-CD133(+/−) cells. The viability of ATC-CD133 cells as determined by the MTT assay significantly decreased as the concentrations of cucurbitacin I increased (p < 0.05) (Fig. 3B). Treatment of ATC-CD133 cells with cucurbitacin I (100 nM) also significantly interfered with sphere formation (Fig. 3C), colony formation (Fig. 3D), and invasion (Fig. 3E). Furthermore, the treatment with 150 nM cucurbitacin I can dramatically suppress the tumorigenic, invasive, and self-renewing abilities of ATC-CD133 cells (p < 0.05) (Fig. 3, B–E). Consistent with these findings, the mRNA levels of stemness genes were significantly reduced after 100 nM cucurbitacin I treatment (p < 0.05) (Fig. 3F). These data suggest that STAT3 may be pivotal for maintaining the cancer stem cell-like characteristics of presumptive ATC CSCs.

**Cucurbitacin I Promotes Differentiation by Blocking STAT3 Signaling in ATC.** Because our data supported the fact that the STAT3 and the p-STAT-related pathway play a crucial role in maintaining the CSC-like property of ATC-CD133 cells, we therefore explored the gene signature profiling of ATC-CD133 cells with or without cucurbitacin I. With the use of gene expression microarray analysis and bioinformatic analysis, the hierarchical heat map of ATC-CD133 cells with or without cucurbitacin I was generated and is shown in Fig. 4A. The expression pattern of ATC-CD133 cells approximated that of embryonic stem cells and mesenchymal stem cells, whereas cucurbitacin I-treated ATC-CD133 cells had profiles that were more similar to those of ATC-CD133 cells (Fig. 4A). Principal component analysis showed that the gene expression profile of cucurbit-
acin I-treated ATC-CD133\(^+\) cells was shifted toward that of ATC-CD133\(^+\) cells (Fig. 4B). These results suggested that the treatment with cucurbitacin I can promote the transcriptional pattern of ATC-CD133\(^+\) cells shifted into the expression profiling of ATC-CD133\(^+\) cells. To further validate the microarray and bioinformatic findings, we examined whether the proportion of CD133\(^+\) cells was suppressed by STAT3 inhibition. First, CD133\(^+\) cells were incubated with 100 or 150 nM cucurbitacin I for 24 h. Fluorescence-activated cell sorter analysis showed that cucurbitacin I decreased the number of ATC-CD133\(^+\) cells in a dose-dependent manner \((p < 0.05)\) (Fig. 4, C and D). Taken together, these results suggest that CD133\(^+\) cells share stem cell-related gene signatures, and cucurbitacin I can further promote ATC-CD133\(^+\) cells differentiated into ATC-CD133\(^+\) cells.

**Cucurbitacin I Restores the Sensitivity of ATC-CD133\(^+\) Cells to Radiochemotherapy.** Lack of NIS, thyroid peroxidase (TPO), and thyroglobulin (Tg) expression in ATC cells makes them resistant to conventional radioiodide therapy (Spitzweg, 2009). Re-expression of these genes in ATC cells has proven to be a promising gene therapy approach. To evaluate the effects of STAT3 abrogation on these thyroid-specific genes, their transcripts were analyzed by quantitative real-time RT-PCR. Addition of 100 or 150 nM cucurbitacin I induced the expression of NIS, TPO, and Tg mRNA in ATC-CD133\(^+\) cells \((p < 0.05)\) (Fig. 5, A–C). The effects of cucurbitacin I on STAT3 downstream genes and cell survival-related genes such as survivin and Bcl-2, as well as on the expression of NIS, were examined by Western blot. Protein levels of Bcl-2 and survivin were down-regulated by cucurbitacin I treatment, whereas NIS production was enhanced (Fig. 5D). In addition, the protein level of MMP-9, which is a key regulator in the metastatic process, declined when STAT3 signaling was abrogated (Fig. 5D). To assess the effects of STAT3 inhibition on iodide uptake, untreated and cucurbitacin I-treated cells were incubated with Na\(^{125}\)I for 24 h. Nonspecific iodide accumulation in the presence of NaClO\(_4\) did not significantly change in untreated cells; however, when CD133\(^+\) cells were treated with cucurbitacin I for 24 h, a marked increase in \(^{125}\)I uptake was observed (Fig. 5, E and F), which was abolished by NaClO\(_4\) (a specific inhibitor of NIS-mediated iodide uptake). In agreement with the microarray and quantitative real-time RT-PCR findings, the \(^{125}\)I uptake assay showed that cucurbitacin I-induced iodide uptake might be facilitated by the differentiation of CD133\(^+\) cells and the subsequent re-expression of functional NIS.

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**Fig. 2.** Microarray analysis of CD133\(^+/−\) cells from ATC tissues. A, hierarchical heat map depicting the gene expression microarray analysis results (gene tree) of the 987 genes that were expressed differentially in ATC-CD133\(^+\) and ATC-CD133\(^−\) cells. B, the literature networks are based on the results from indexing using a natural language processing regimen of all MEDLINE records (titles and abstracts) for gene and protein names. STAT3 is the major hothub of ATC-CD133\(^+\) for linking the gene network. Lines indicate co-citation in the literature in more than one article.
Cucurbitacin I Sensitizes ATC-CD133 Cells to Radiochemotherapy. To examine the influence of cucurbitacin I on the chemoresistance of ATC-CD133 cells, cytotoxicity analysis was performed. Cucurbitacin I treatment of ATC-CD133 cells rendered them more sensitive to the chemotherapeutic agents cisplatin (Fig. 6A), 5-fluorouracil (Fig. 6B), and doxorubicin (Fig. 6C), all of which are currently used for thyroid cancer chemotherapy. In addition, sphere formation (Fig. 6D), colony formation (Fig. 6E), and invasiveness (Fig. 6F) were dramatically reduced in the cells treated with chemotherapeutic agents plus cucurbitacin I compared with reduction in cells treated with either agent alone.

We next examined whether the radioresistance of CD133+ cells was due to modulation of STAT3 signaling. Vehicle- or cucurbitacin I-treated ATC-CD133 cells and CD133- cells were exposed to different doses of ionizing radiation. Cellular survival fraction analysis indicated that CD133+ cells were more radioresistant than CD133- cells (Fig. 7A). Of note, silencing of STAT3 signaling with cucurbitacin I sensitized CD133+ cells to radiation. Cucurbitacin I in combination with 4 Gy IR dramatically diminished sphere formation (Fig. 7B) and colony formation and invasion (data not shown) in both CD133+ cells and CD133- cells. Based on clinical observations, the propensity to metastasize is one of the major causes of the poor prognosis associated with ATC. To investigate whether cucurbitacin I treatment could suppress ATC cell migration, cell mobility assays were performed with both CD133+ cells and CD133- cells treated with cucurbitacin I and/or IR. Compared with the control group, a single treatment with cucurbitacin I or IR reduced cell mobility; a more prominent reduction in cell mobility was observed after the cells were treated with cucurbitacin I plus IR (Fig. 7C).
Moreover, caspase 3 activity analysis revealed that the cytotoxic effect of IR on ATC-CD133 cells was significantly enhanced with the addition of cucurbitacin I (p < 0.05) (Fig. 7D). In addition, terminal deoxynucleotidyl transferase dUTP nick-end labeling-positive cells concomitantly increased among ATC-CD133 cells treated with cucurbitacin I and IR compared with cells treated with either method alone (Fig. 7E). These results suggest that cucurbitacin I can greatly increase the effectiveness of radiotherapy against ATC-CD133 cells by driving apoptosis.

Effects of Cucurbitacin I on the In Vivo Tumorigenicity and Survival Time of ATC-CD133 Cells in a Xenotransplantation Animal Model. We next investigated the role of STAT3 signaling and the effects of cucurbitacin I in ATC-CD133 cells in vivo. ATC-CD133(+/−) cells were transfected with a lentiviral vector containing the GFP gene. We injected 2 × 10⁶ CD133-GFP cells and 2 × 10⁶ CD133-GFP cells into the subcutaneous regions of nude mice that had received different treatment protocols (Fig. 8A). We found that the subset of nude mice receiving CD133-GFP cells had formed no tumors in the subcutaneous region by 10 weeks after transplantation. CD133-GFP cells presented the greatest ability to form tumors in transplanted mice (Fig. 8A). Mice bearing subcutaneous tumors received cucurbitacin I (1 mg/kg i.p. for 5 days) or cisplatin plus 4 Gy IR. Both treatment strategies suppressed the proliferation of CD133-GFP cells in the transplanted mice (Fig. 8A). Of note, the combination triple treatment (cucurbitacin I + cisplatin + IR) caused a significant amount of tumor shrinkage in CD133-GFP cell-transplanted mice (p < 0.05) (Fig. 8A).
To confirm that STAT3 was crucial for metastasis in vivo, mice were orthotopically injected with either CD133+/H11001-GFP or CD133+/H11002-GFP cells. After transplantation, CD133+/H11001 cells showed significantly higher levels of local invasion and distant metastasis to the lungs than CD133+/H11002 cells, and they formed larger tumors (Fig. 8B). Either strategy (cucurbitacin I alone or cisplatin plus IR) modestly decreased local invasion and distant metastasis in a statistically significant manner. Most importantly, the triple treatment (cucurbitacin I plus cisplatin plus IR) of CD133+/H11001 cells effectively reduced the number of lung metastases and volumes in vivo (Fig. 8B). Moreover, CD133+/H11001 cell-transplanted mice treated with the triple treatment had a mean survival rate that was significantly prolonged compared with that of CD133+/H11001 cell-transplanted mice that had received other treatments (Fig. 8C). Overall, these in vivo results show that the effectiveness of conventional chemoradiotherapy in mice bearing ATC-CD133+/H11001 tumors can be significantly improved with the addition of cucurbitacin I treatment.

Discussion

Most well differentiated thyroid cancers of follicular cell origin have good prognosis with a 10-year survival rate in the literature of greater than 85% (Sipos and Mazzaferri, 2010). However, for patients with ATC with metastasis to distant organs and for those with postoperative recurrence or lymphoid metastatic lesions without radioiodine uptake, the success of therapy is limited and the cancer may be life-threatening. At present, neither surgery nor chemoradiation is an effective treatment for ATC with respect to achieving long-term survival. Rapid local recurrence and distant metastasis with resistance to chemotherapy or radiotherapy are the major causes of treatment failure (Cornett et al., 2007).
the increasing awareness of the parallels between stem cells and cancer cells, evidence supporting the existence of CSCs that contribute to tumorigenicity has been accumulating (Jordan et al., 2006). A current approach to enriching for CSCs is the use of specific cell surface markers combined with cell sorting. CD133, a 5-transmembrane glycoprotein, is a hematopoietic stem cell and endothelial progenitor marker that seems to be involved in angiogenesis (Corbeil et al., 2000; Hilbe et al., 2004). Recent studies have identified a subpopulation of CD133\(^+\)/H11001 cells in ATC cell lines that exhibit definitive properties of cancer stem cells: self-renewal, multipotency, and tumorigenicity in vitro or in vivo (Zito et al., 2008; Friedman et al., 2009). The expression of Oct4 has been found to be consistently up-regulated in CD133\(^+\) cells, suggesting the Oct4, an embryonic stem cell gene, plays a critical role in thyroid CSCs. Moreover, an immunohistochemical analysis of ATC tissue specimens performed by Friedman et al. (2009) showed that tumor cells expressed CD133, whereas neighboring normal thyroid cells did not, suggesting that CD133 could be a therapeutic target in ATC.

However, the regulatory mechanisms active in ATC CSCs have not been clarified, impeding the subsequent development of potential therapies against these cells. In this study, we showed that relative to CD133\(^+\)/H11002 cells, CD133\(^+\)/H11001 cells exhibited greater CSC-like properties such as increased tumorigenicity and expression of embryonic stemness and drug-resistant genes (Fig. 1). Microarray and bioinformatic analyses indicated that the STAT3 and p-STAT3 signaling axis is an important link between the tumorigenic and stemness gene expression profiles and the metastasis-associated pathways in ATC CSCs (Fig. 2). Exploiting this finding, we next demonstrated that 100 nM cucurbitacin I, a specific JAK-STAT inhibitor, efficiently inhibits the proliferation of ATC-CD133\(^+\) cells and induces apoptosis in them (Figs. 3 and 7). Furthermore, 100 nM cucurbitacin I was found to inhibit the stemness gene signatures of ATC-CD133\(^+\) cells and facilitate their differentiation into ATC-CD133\(^-\) cells (Figs. 3 and 4). Moreover, blocking STAT3 signaling with cucurbitacin I significantly suppresses the self-renewal ability, tumorigenicity, and radiochemoresistance of ATC-
CD133^+ cells, suggesting that STAT3 activation plays a role in maintaining their CSC phenotype (Figs. 6 and 7). Finally, Kaplan-Meier survival analysis indicated that the effect of cisplatin or 4 Gy IR on ATC-CD133^+ cells is significantly enhanced by cucurbitacin I treatment (Fig. 8). To our knowledge, this is the first study to not only show that the STAT3 axis plays an important role in maintaining CSC-like properties but also demonstrate that targeting STAT3 with cucurbitacin I significantly suppresses tumorigenicity and radiochemoresistance in ATC-derived CSCs.

The NIS is a central mediator of active iodide transport into the thyroid gland (Carvalho and Ferreira, 2007). Because of its key role in thyroid physiology and pathophysiology, both re-expressing endogenous NIS and introducing exogenous NIS have become candidate anticancer strategies. In addition to gene therapy, several compounds have been found to harbor an ability to induce endogenous NIS gene expression. Retinoic acid was shown to trigger redifferentiation of thyroid cancer cell lines and up-regulate NIS expression (Riesco-Eizaguirre and Santisteban, 2006; Carvalho and Ferreira, 2007). A number of clinical trials using retinoic acid have been performed but have shown variable results. Because Pax8-PPARγ rearrangement is a common occurrence in thyroid cancers (Marques et al., 2002; Castro et al., 2006), PPARγ may be involved in thyroid carcinogenesis. Troglitazone, a PPARγ agonist, has been reported to induce differentiation and NIS mRNA expression in human thyroid cancer cell lines (Park et al., 2005). Another emerging class of pharmacological agents belongs to the group of histone deacetylase inhibitors, which are used to re-express silenced genes in

Fig. 7. Cucurbitacin I increases chemosensitivity and synergistically suppresses tumor sphere formation by inducing apoptosis in CD133^+ cells. A, to determine the effect of radiation on tumor growth rate, IR doses from 0 to 10 Gy were used to treat CD133^+ and CD133^- cells in combination with vehicle or cucurbitacin I. B, the sphere formation ability of CD133^+ and CD133^- cells was examined after treatment with cucurbitacin I, 4 Gy IR, or both. Scale bar, 100 μm. C, top, representative pictures of mobility assays of cells treated with dimethyl sulfoxide (control) or 100 or 150 nM cucurbitacin I for 24 h. Bottom, percentage of mobile CD133^+ and CD133^- cells treated with different concentrations of cucurbitacin I and/or IR. Cleaved caspase 3-positive cells (D) and terminal deoxynucleotidyl transferase dUTP nick-end labeling-positive cells (E) were determined among CD133^+ and CD133^- cells after treatment with IR in the presence or absence of cucurbitacin I. Data shown are the mean ± S.D. of three independent experiments. *, p < 0.05.
cancer cells. Depsipeptide (FR901228), trichostatin A, and valproic acid have been shown to successfully restore the expression of NIS (Furuya et al., 2004; Riesco-Eizaguirre and Santisteban, 2006). Moreover, STAT3, a transcription factor regulated by various cytokines and growth factors (particularly interleukin-6 and EGF) is aberrantly activated in numerous cancers (Bollrath and Greten, 2009; Yu et al., 2009). Several cytokines such as interleukin-6 have proven to suppress NIS mRNA levels in the Fisher rat thyroid cell line (FRTL-5) (Spitzweg et al., 1999; Riesco-Eizaguirre and Santisteban, 2006). In a more recent study, treatment with sunitinib, a selective inhibitor of receptor tyrosine kinases, seemed to block STAT3 activation and induce the expression of NIS in papillary thyroid cancer (Fenton et al., 2010). In the present study, we found that treating ATC-CD133+ cells with cucurbitacin I up-regulates their expression of thyroid-specific genes including sodium/iodide symporter, thyroperoxidase, and thyroglobulin, and it significantly enhances NIS-mediated radioiodine uptake (Fig. 5). Of importance, our data suggest that the abrogation of the STAT3 signaling pathway induces the expression of NIS protein and subsequently augments radioiodide accumulation, indicating that STAT3 may be a regulator of NIS (Fig. 5). Therefore, even though the biomolecular links between the STAT3 axis pathways and NIS remain unclear, the finding that a STAT3 inhibitor induces the expression of thyroid-specific genes should facilitate the development of novel strategies against thyroid cancer.

The CSC hypothesis has been bolstered by the clinical observation that malignant tumors are relatively resistant to chemoradiotherapies (O’Brien et al., 2010). The existence of cancer stem cell-like cells may explain why conventional...
anticancer therapies can only partially eradicate it, resulting in eventual recurrence (Singh and Settleman, 2010). Consistent with this hypothesis, ATC-CD133+ cells are significantly more resistant to cisplatin, 5-FU, and doxorubicin than ATC-CD133− cells (Figs. 1 and 6). IR alone and single chemotherapeutic drugs were able to effectively inhibit the growth of ATC-CD133+ cells (Fig. 8), whereas IR treatment combined with cisplatin still failed to cause death in ATC-CD133− cells (Fig. 8).

To overcome the resistance to radiotherapy and chemotherapy of ATC-CD133+ cells, cucurbitacin I treatment was used to abrogate STAT3 signaling in them. When combined with other treatments, cucurbitacin I significantly improved their in vivo anticancer effects (Fig. 8). Our data also suggest that cucurbitacin I significantly induced apoptosis in ATC-CD133+, in part through the inactivation of p-STAT3 and its downstream effectors survivin, Bcl-2, and MOMP (Fig. 5). Furthermore, our data suggest that inhibition of the STAT3 pathway by cucurbitacin I significantly blocked the migration and lung metastasis of ATC-CD133+ cells in both in vitro and in vivo xenotransplantation models (Figs. 7 and 8). Of note, in vivo xenotransplantation analysis showed that cucurbitacin I can be a sensitizer synergistically enhancing both radio- and chemosensitivity in a xenotransplantation model of human thyroid cancer stem cells (Fig. 8). Taken together, our findings indicate that the antiproliferative, proapoptotic, and radiochemosensitizing effects of cucurbitacin I on CD133+ cells could be used as a strategy to overcome the resistance of highly tumorigenic ATC-CD133+ cells in patients with advanced ATC. Treating ATC by targeting JAK/STAT3 signaling together with other components of the CSC-related microenvironment that biologically affect radiochemoresistance, recurrence, and metastasis should be further investigated.

In conclusion, we showed that the STAT3 signaling axis may be responsible for the CSC-like properties and radioresistance of ATC-CD133+ cells. Cucurbitacin I potently attenuated the malignancy of ATC-CD133+ cells, presenting a potential clinical benefit for thyroid cancer and CSC treatment. Thus, there is a great need to determine the underlying mechanisms of the STAT3 pathway in ATC-CD133+ cells and to further evaluate the therapeutic potential of STAT3 pathway inhibitors. In addition, persistent activation of STAT3 promotes tumor cell proliferation and survival, contributing to tumor progression and migration. Therefore, abrogation of STAT3 signaling may be a potent therapeutic strategy for malignant thyroid cancers as well as cancer stem cells in general.

Authorship Contributions
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References


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