HIF1α in Tumorigenesis of Adenoid Cystic Carcinoma

YUN-SUNG LIM1, WONJAE CHA2, MIN-WOO PARK3, WOO-JIN JEONG4 and SOON-HYUN AHN4

1Department of Otorhinolaryngology-Head and Neck Surgery, Ilsan Hospital, Dongguk University, Goyang, Republic of Korea; 2Department of Otorhinolaryngology-Head and Neck Surgery, Pusan National University School of Medicine, Busan, Republic of Korea; 3Department of Otorhinolaryngology-Head and Neck Surgery, Ilsong Memorial Institute of Head and Neck Cancer, Hallym University, College of Medicine, Seoul, Republic of Korea; 4Department of Otorhinolaryngology-Head and Neck Surgery, Seoul National University College of Medicine, Seoul National University Bundang Hospital, Seongnam, Republic of Korea

Abstract. Background: Tumor hypoxia induces hypoxia-inducible factor-1α (HIF1α), which can influence tumorigenesis and metastasis. We evaluated the expression of HIF1α and the effect of HIF1α inhibitors in adenoid cystic carcinoma (ACC). Materials and Methods: HIF1α expression was demonstrated in ACC cell lines (ACC2 and ACCM). The effect of HIF1α inhibitors was evaluated. A systemic metastasis model was developed. The number of metastatic pulmonary nodules were analyzed. Results: The ACCM cell line demonstrated greater HIF1α expression and invasion than ACC2. The expression of HIF1α and invasion of ACC cells were blocked by HIF1α siRNA. HIF1α inhibitors 17-N-allylamino-17-demethoxygeldanamycin (17AAG) and echinomycin inhibited cell invasion. 17AAG inhibited metastasis in the animal model, although not statistically significantly. Conclusion: HIF1α siRNA and 17AAG and echinomycin blocked invasion by ACC2 and ACCM cells. 17AAG exhibited therapeutic potential for inhibition of metastasis. Our results provide positive evidence that HIF1α is a promising research pathway for therapy of ACC.

Adenoid cystic carcinoma (ACC) is a common malignancy of the salivary glands, representing 10% of all salivary gland neoplasms (1). ACC is a slow-growing but highly malignant tumor because it is characterized by intensive local invasion and insidious distant metastasis to the lung at an early stage (2). It has an aggressive clinical course and usually results in a very poor long-term outcome: only 20% of patients diagnosed with distant metastasis survive 5 years (3).

A characteristic of tumorigenesis, as well as several other physiological processes, is a reduction of oxygen tension in tissues, leading to a state of hypoxia. This condition limits the growth of tumors if blood vessels are remote.

Because hypoxia generates an unfavorable situation for cell growth, cancer cells undergo a series of genetic and metabolic changes that allow them to survive and even proliferate by acquisition of the ability to recruit their own blood supply system under hypoxic stress (4). In the clinical setting, hypoxia is associated with malignant progression, and resistance to radiotherapy and chemotherapy (5). The risk of metastasis is higher with hypoxic tumors, not only for head and neck squamous tumors, but also for cervical and breast cancer (6-8).

Hypoxia-inducible factor-1 (HIF1) has been extensively studied as the most important factor involved in the cellular response to tumor hypoxia (9). Under hypoxic conditions, it regulates genes that enable survival by binding to hypoxia-response elements (10). These include those encoding erythropoietin, glucose transporters, vascular endothelial growth factor, heme oxygenase and inducible nitric oxide synthase (11).

HIF1 is a heterodimeric basic helix-loop-helix Per-ARNT-Sim (PAS) domain protein with two components, an α subunit that is regulated by cellular oxygen tension, and a β portion that is expressed constitutively (12). Overexpression of HIF1α is an early event in prostate carcinogenesis and is predictive of early relapse in breast cancer (13, 14). A common cellular protein, heat-shock protein (HSP90), regulates the physiological response to environmental stress but as an unfortunate side-effect can maintain malignant transformation. Thus, blocking HSP90 will degrade in a proteasome-dependent fashion those client
proteins (e.g. HIF1α, p53, BRAF and BCR-ABL fusion protein) which are relevant cancer targets (15).

The poorly metastatic cell line ACC2 was established from ACC, and the highly metastasis cell line ACCM is a clone highly metastatic to the lung selected from ACC2 (16). The metastatic rate was 96% for ACCM and 18% for ACC2 by injection in the tail vein of BALB/c nude mice (16). In the present study, we examined the expression of HIF1α in these cell lines in vitro and its inhibition by blockage of the HIF pathway. We also developed a model of hematogenous pulmonary metastasis in nude mouse by cell line implantation to identify the role of HIF1α in metastasis of ACC. Furthermore, we investigated the role of 17-N-allylamino-17-demethoxygeldanamycin (17AAG) and echinomycin, inhibitors of HIF1α, in vitro and in vivo as molecular targeted agents in this pulmonary metastasis model.

Materials and Methods

Cell line, culture, and preparation. Human ACC cell lines were a gift from Shanghai Second Medical University, Shanghai, China. Cells were cultured for 3 days in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1xnon-essential amino acids and antibiotics in an incubator at 37°C with 5% CO₂.

Compounds. In order to assess the inhibition of HIF1α expression, ACC cell lines were treated with the HIF1α inhibitors 17AAG (0.025 and 0.05 µM; Tocris Bioscience, Bristol, UK), echinomycin (0.0001-1 µM; Santa Cruz Biotechnology, Dallas, TX, USA), KC7F2 (0.1-1,000 µM; Tocris Bioscience), LAQ824 (0.001-1 µM; Dacrinostat; Selleckchem, Houston, TX, USA), temsirolimus (0.1-1,000 µM; LC Laboratories, Woburn, MA, USA), vorinostat (0.1-1,000 µM; LC Laboratories) and YC1 (0.001-1,000 µM; Tocris Bioscience). HIF1α inhibitors were dissolved in dimethyl sulfoxide (DMSO) and diluted with RPMI-1640 medium to the desired concentration for in vitro experiments. Cell invasion and proliferation assays were carried out in treated ACC2 and ACCM cell lines.

Immunoblotting. Buffer for extracting cytoplasm protein (10 mM HEPES, pH 7.9, 10 mM KCl, 1 mM dithiothreitol (DTT) and 0.5 mM phenylmethylsulfonyl fluoride (PMSF); 0.1 mM EDTA) was applied to untreated and control siRNA- and HIF1α siRNA-treated cells. The cells were detached from the plate after mixing in a shaker. Following incubation on ice for 15 min, samples were centrifuged at 15,000 x g for 10 min and the cytoplasmic protein was extracted. A buffer for extracting nuclear protein (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT and 1 mM PMSF) was applied to the pellet which was then incubated for 5 minutes on ice. The samples were centrifuged at 15,000 x g for 10 minutes and the nuclear proteins were extracted. Western blotting was performed using anti-HIF1α (Cell Signaling Technology, Danvers, MA, USA) and anti-glyceraldehyde phosphate dehydrogenase (GAPDH; Sigma, St Louis, MO, USA).

Proliferation assay. ACCM cells (2x10⁵) were plated in 96-well plates in triplicate and incubated for 3 days. Control siRNA, HIF1α siRNA, 17AAG, echinomycin, KC7F2, LAQ824, temsirolimus, vorinostat and YC1 were administered at different concentrations along with control wells. Cell proliferation assay was performed using a cell counting kit-F (CCK-F) from Dojindo Molecular Technologies (Gaithersburg, MD, USA). A total of 1x10⁵ cells were loaded in serum-free medium and 10 µl of CCK-F solution was applied to the well. After 2 h incubation, the absorbance at a wavelength of 450 nm was recorded using a Synergy HT multidection microplate reader (BioTek Instruments, Winooski, VT, USA). The effect of HIF1α siRNA and HIF pathway inhibitors on proliferation of ACC cell lines was evaluated. The IC₅₀ value, i.e. the concentration at which 50% of the cell growth is inhibited compared with the control, was calculated by nonlinear regression analysis using GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA).

Invasion assay. The effect of HIF1α siRNA and HIF pathway inhibitors 17AAG, echinomycin on cell invasion of ACC cell lines was evaluated in Boyden chambers fitted with Matrigel coated filters (Matrigel invasion chambers; BD Biocoat, San Jose, CA, USA). A total of 1x10⁵ cells were loaded in serum-free medium into the top wells of the Boyden chamber and normal media containing as a chemotactrant in the bottom well. In the top well, cells were treated with inhibitors. After incubation for 22 h, the cells invaded to the other side of the filter were fixed and stained with Harleco Hemacolor staining kit (EMD Chemicals, Gibbstown, NJ, USA). Cells were counted at ×200 in three different fields from each well. The experiments were performed in triplicate and the results reflect the outcome of at least three independent experiments.

Animal model. A lung metastasis model was developed in 15, 8- to 12-week-old female BALB/c nude mice (Orient Bio, Seongnam, Gyeyonggi, Korea) An incision of approximately 1 cm over the skin surface of the neck was made and the internal jugular vein was exposed then 1x10⁶ cells in 0.1 ml volume were injected into the vein and the skin was closed. Five mice were randomly assigned to two treatment and a control groups.
For the treatment groups, two different kinds of HIF pathway inhibitor, 17AAG and echinomycin, were used. Following the injection of tumor cells, mice were injected with HIF pathway inhibitor on every weekday for 2 weeks. 17AAG and echinomycin were injected intraperitoneally at 50 mg/kg and 0.15 mg/kg, respectively, in 10% DMSO. Mice in the control group were injected with the same volume of 10% DMSO.

On day 42, mice were sacrificed and the lungs were inspected grossly. After extraction of the lungs, specimens were fixed with 10% formalin solution and the numbers of metastatic foci on the lung surface were counted. The animal study was performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee (no. 63-2010-027).

Statistical analysis. SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA) was used. Fisher’s exact test and the Mann–Whitney U-test were used to compare groups.

Results

HIF1α expression and invasion assay by ACC cells. Western blotting showed increased HIF1α expression in the nucleus of ACC cells (Figure 1A). qRT-PCR showed that the expression of HIF1α was 1.85-times higher in ACCM cells than in ACC2 cells (Figure 1B). The invasion assay demonstrated that ACCM cells have greater invasive ability than ACC2 cells, although not statistically significantly so (Figure 1C and D).

siRNA knockdown of HIF1α. HIF1α expression was blocked by HIF1α siRNA in a dose-dependent manner (Figure 2A). In the proliferation assay, HIF1α siRNA demonstrated a growth-inhibitory effect on ACCM cells in vitro, although not statistically significant (Figure 2B). The invasion assay using HIF1α siRNA found that invasion of ACC2 and ACCM cells was significantly compromised ($p=0.018$ and $p<0.0001$) (Figure 2C and D).

Inhibition of ACC cells by HIF1α inhibitors. The proliferation assay showed the different HIF pathway inhibitors inhibited growth in the ACCM cell line. The IC$_{50}$s were: echinomycin: 0.00046 μM, KC7F2: 44.53 μM, LAQ824: 44.53 μM, temsirolimus: 29.61 μM, vorinostat: 0.094 μM, and YC1: 3782 μM (Figure 3A). 17AAG at 0.025 μM and 0.05 μM did not significantly inhibit the growth of ACCM cells.
not inhibit growth of ACC2 and ACCM cells, but 0.0006 μM of echinomycin did in ACC2 cells, although not statistically significantly (Figure 3B).

Invasion by ACC2 and ACCM cells was significantly reduced by echinomycin as well as 17AAG compared to the control (p<0.05) (Figure 3C).

Inhibition of ACC lung metastasis by 17AAG. A lung metastasis model was successfully developed by systemic injection of ACCM cell line into nude mice (Figure 4A). Microscopically, multiple metastatic tumor foci can be seen in Figure 4B. 17AAG and echinomycin were injected every weekday until 2 weeks after injection of tumor cells (Figure 4C). Administration of 17AAG reduced the number of metastatic foci compared to the control group (mean number: 59.3 vs. 39.9), although this was not statistically significant (p>0.05) (Figure 4D). Echinomycin also did not result in a significant difference infrequency of metastatic foci.

Discussion

This study demonstrated that HIF1α is expressed in ACC cell lines, and overexpressed in a highly metastatic ACC line which has a more invasive phenotype. Our results also revealed inhibition of expression of HIF1α by HIF1α siRNA and blockade of invasion of ACC2 and ACCM cells by HIF1α siRNA, 17AAG, and echinomycin. Lung metastasis successfully developed in a systemic metastasis model in nude mice. 17AAG demonstrated therapeutic potential for inhibition of tumorigenesis at metastatic foci. The HIF1α inhibitors 17AAG and echinomycin were applied to this model for the first time as far as we know.
The tumor microenvironment can be described as both oxygen- and nutrient-starved. Oxygen tension in normal tissue averages 7%, while that in tumors is 1.5% (17, 18). By adapting to the low pO₂ or by increasing vascularization, tumor cells survive. Clones adapt both genetically and by methylation, gaining invasiveness and metastatic capability by loss of cell-cycle control, loss of normal apoptotic signals, genetic instability, and aerobic glycolysis. As a result, human
cancer clones with high lethal potential are adaptable to hypoxia both at primary and metastatic sites.

Traditional therapies are challenged by tumors that adapt to hypoxia. Radiotherapy is compromised because of the reduced reaction of oxygen with DNA free radicals (19). The remoteness from blood vessels limits delivery of chemotherapeutic agents to hypoxic sites. Moreover, many anticancer drugs are most effective against rapidly proliferating cells, and hypoxia can cause a reduction in the rate of cell proliferation (20). For the same reason that chemotherapy is hampered, immune response is impaired at hypoxic sites, allowing cancer stem cells to develop (21, 22). Therefore, therapeutic modalities that attack such hypoxic regions are a necessity.

The synthesis of factors promoting cancer cell survival and proliferation is driven by an HIF transcriptional pathway which is activated in hypoxia. HIF1α is a potential prognostic biomarker in proteomic assessments of breast and prostate cancer (13, 14). Schrijvers et al. reported that HIF1α overexpression in early-stage glottic carcinoma was significantly associated with worse local control and overall survival, and that tumors with a non-hypoxic profile had significantly better local control (23). Kyzas et al. reported that HIF1α and VEGF expression was significantly associated with tumors located in the lower lip and larynx (24).

In addition, the expression of HIF1α is increased in papillary thyroid carcinoma (PTC), the most common type of head and neck cancer. Scarpino et al. reported that the levels of HIF1α from PTC were consistently higher (4.5±3-fold) in tumor compared to the corresponding normal thyroid tissue (25). To our knowledge, there are few reports on the expression of HIF1α in patients with ACC. Costa et al. reported that HIF1α expression is a common finding in

---

**Figure 4. Lung metastasis model and inhibition by hypoxia-inducible factor-1α (HIF1α) inhibitors.** Multiple metastatic nodules are visible on gross (A) and histological (B) examination (staining, ×100). C: Animal study protocol. 17-N-Allylamino-17-demethoxygeldanamycin (17AAG) reduced the number of metastatic foci (D). W: Weeks.
ACC (26). Hara et al. reported that hypoxia activates the HGF/c-Met system via HIF1α expression in ACC cell line, and that it might be involved in metastasis in human salivary gland cancer (27). Despite good local control, about 40-60% of patients with ACC suffer from distant metastases (lungs, bones, and soft tissues) (2). Sung et al. reported disease-specific 5-year survival rate of 88%, and 10-year rate of 72% for patients without distant metastasis, but rates of 76% and 48%, respectively, for those with distant metastasis (2).

With unique biological behavior, the precise characteristics of metastasis are not fully understood. In this context, the study of the expression of HIF1α in ACC in vitro and in vivo models for distant metastasis using highly and poorly metastatic ACC cell lines is necessary, in that targeting the HIF pathway as a promising anticancer strategy can give much aid for patients suffering from ACC with distant metastasis.

Ahn et al. reported pre- and postoperative echinomycin treatment significantly improved post-surgery survival and recurrence in an animal model of tongue cancer (28). Mo et al. reported that HIF1α siRNA and 17AAG blocked the invasiveness of BHP10-3SC (a tumorigenic subclone from human PTC cell lines BHP10-3) by inhibition of HIF1α expression in vitro and in the orthotopic PTC animal model (29). These are the fundamental reasons we selected echinomycin and 17AAG as the inhibitors of HIF pathway.

Echinomycin, a cyclic peptide of the family of quinoxaline antibiotics originally isolated from Streptomyces echinatus, is a small molecule that binds DNA in a sequence-specific fashion (30). The strong binding sites for echinomycin contain the central 2-bp sequence 5V-CG-3V, and the key recognition elements for echinomycin are contained in the sequences 5V-ACGT-3V and 5V-TCGT-3V (31). Electrophoretic mobility shift assay showed that echinomycin has HIF1 inhibitor activity by binding to the hypoxia response elements which HIF1α and HIF1β proteins bind to and contain the 5V-ACGT-3V sequence (32). Although it had the lowest IC50 among the various HIF pathway inhibitors and blocked the invasion of ACC2 and ACCM cells significantly in vitro, it did not inhibit tumorigenesis and tumor growth in the ACC lung metastasis model. Vlaminck et al. reported that it cannot be used in cancer treatment because of its dual effect on HIF1 activity under normoxic and hypoxic conditions (33). Echinomycin strongly blocks HIF1 activity in hypoxia and interferes with the activity of other transcription factors. Under normoxic conditions, echinomycin increases the activity of HIF1, with a concomitant increase in expression of HIF1 target genes. These findings might explain our findings that injection of echinomycin did not result in significant change because the lung is one of the most normoxic organs in the body.

17AAG inhibits HIF1α by specifically blocking the chaperone protein HSP90. Our data showed that treatment with 17AAG inhibited the invasion of ACC2 and ACCM cells significantly in vitro and tumorigenesis at metastatic foci in vivo in this ACC lung metastasis model, despite not reaching statistical significance. We assume that these findings originate from the differences in the microenvironment between in vitro and in vivo study and to the relatively a small number of animals.

**Conclusion**

This study demonstrates that there is a good association between tumorigenic potential and nuclear HIF1α expression in the cell lines tested, and that HIF1α is important in metastatic tumor growth in ACC. HIF1α siRNA and 17AAG and echinomycin blocked the invasion of ACC2 and ACCM cells, and 17AAG demonstrated therapeutic potential in inhibiting tumorigenesis at metastatic sites. Our results suggest promising research paths, based on the potential of the biologically-relevant HIF1α target molecule as an agent in chemoradiotherapy for ACC, or as an adjunct to prevent recurrence.

**Conflicts of Interest**

The Authors have no conflicts of interest to disclose.

**References**


Received December 7, 2016
Revised January 17, 2017
Accepted January 18, 2017