HIF1 α in Tumorigenesis of Adenoid Cystic Carcinoma

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Abstract. *Background: Tumor hypoxia induces hypoxia-inducible factor-1 α (HIF1 α), which can influence tumorigenesis and metastasis. We evaluated the expression of HIF1 α and the effect of HIF1 α inhibitors in adenoid cystic carcinoma (ACC). Materials and Methods: HIF1 α expression was demonstrated in ACC cell lines (ACC2 and ACCM). The effect of HIF1 α inhibitors was evaluated. A systemic metastasis model was developed. The number of metastatic pulmonary nodules were analyzed. Results: The ACCM cell line demonstrated greater HIF1 α expression and invasion than ACC2. The expression of HIF1 α and invasion of ACC cells were blocked by HIF1 α siRNA. HIF1 α inhibitors 17-N-allylamino-17-demethoxygeldanamycin (17AAG) and echinomycin inhibited cell invasion. 17AAG inhibited metastasis in the animal model, although not statistically significantly. Conclusion: HIF1 α 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 HIF1 α 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 HIF1 α 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

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proteins (e.g. HIF1 α , 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 HIF1 α 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 HIF1 α in metastasis of ACC. Furthermore, we investigated the role of 17-*N*-allylamino-17-demethoxygeldanamycin (17AAG) and echinomycin, inhibitors of HIF1 α , *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, 1 $\times$ non-essential amino acids and antibiotics in an incubator at 37°C with 5% CO₂. Western blotting, quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) and invasion assay were performed on ACC2 and ACCM cell lines.

Compounds. In order to assess the inhibition of HIF1 α expression, ACC cell lines were treated with the HIF1 α inhibitors 17AAG (0.025 and 0.05 μ M; Tocris Bioscience, Bristol, UK), echinomycin (0.0001-1 μ M; Santa Cruz Biotechnology, Dallas, TX, USA), KC7F2 (0.1-1,000 μ M; Tocris Bioscience), LAQ824 (0.001-1 μ M; Dacinostat; Selleckchem, Houston, TX, USA), temsirolimus (0.1-1,000 μ M; LC Laboratories, Woburn, MA, USA), vorinostat (0.1-1,000 μ M; LC Laboratories) and YC1 (1-10,000 μ M; Tocris Bioscience). HIF1 α 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 cells.

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 HIF1 α 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 $\times$ g for 10 min and the cytoplasm 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 $\times$ g for 10 minutes and the nuclear proteins were extracted. Western blotting was performed using anti-HIF1 α (Cell Signaling Technology, Danvers, MA, USA) and anti-glyceraldehyde phosphate dehydrogenase (GAPDH; Sigma, St Louis, MO, USA).

qRT-PCR. RNA was isolated using an RNeasy Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. We synthesized single-strand DNA for 50 min at 50°C with oligo-dT primer using reagents in the ThermoScript RT-PCR System (Invitrogen, Rockville, MD, USA). RT-PCR was performed using SYBR Green PCR Master Mix on an ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA). PCR was performed using the following primers: HIF1A forward: GGTTCACCTTTTCAAGCAGTAGG and reverse: TGGTAATCC ACTTTCATCCATTG. A comparative CT method was used to compare expression.

Gene knockdown using siRNA. HIF1 α siRNA (siRNA no.1068432) and control siRNA were purchased from Bioneer (Daejeon, Korea). We used DharmaFECT 4 to perform siRNA-mediated gene knockdown following instructions from Dharmacon (Chicago, IL, USA). We harvested the cells after 24 h of incubation. The inhibitory effects of siRNA on HIF1 α expression and proliferation of ACCM were evaluated by immunoblotting and cell proliferation assay. Invasion assay was performed on ACC2 and ACCM cell lines treated with control siRNA and HIF1 α siRNA.

Proliferation assay. ACCM cells (2 $\times$ 10³) were plated in 96-well plates in triplicate and incubated for 3 days. Control siRNA, HIF1 α 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). For spectrophotometry, after removing the media, 100 μ l of serum-free medium and 10 μ l 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 multidetection microplate reader (BioTek Instruments, Winooski, VT, USA). The effect of HIF1 α 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 HIF1 α 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 1 $\times$ 10⁵ cells were loaded in serum-free medium into the top wells of the Boyden chamber and normal media containing as a chemoattractant 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 $\times$ 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 using ACCM was developed in 15, 8- to 12-week-old female BALB/c nude mice (Orient Bio, Seongnam, Gyeonggi, Korea). An incision of approximately 1 cm over the skin surface of the neck was made and the internal jugular vein was exposed then 1 $\times$ 10⁶ 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.

Figure 1. Hypoxia-inducible factor-1 α (HIF1 α) protein (A) and mRNA (B) expression and invasion by adenoid cystic carcinoma (ACC) cell lines. Western blot showed increased HIF1 α expression in the nucleus of ACC cells (A). ACCM cells demonstrated 1.85-fold higher expression of HIF1 α than ACC2 (B). ACCM cell line was found to have non-significantly greater invasive ability than ACC2 (C and D).

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

HIF1 α expression and invasion assay by ACC cells. Western blotting showed an increased expression of HIF1 α in the nucleus of ACC cells (Figure 1A). qRT-PCR showed that the

expression of HIF1 α 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 HIF1 α . HIF1 α expression was blocked by HIF1 α siRNA in a dose-dependent manner (Figure 2A). In the proliferation assay, HIF1 α siRNA demonstrated a growth-inhibitory effect on ACCM cells *in vitro*, although not statistically significant (Figure 2B). The invasion assay using HIF1 α 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 HIF1 α inhibitors. The proliferation assay showed the different HIF pathway inhibitors inhibited growth in the ACCM cell line. The IC₅₀s were: echinomycin: 0.00046 μ M, KC7F2: 44.53 μ M, LAQ824: 44.53 μ M, temsirolimus: 29.61 μ M, vorinostat: 0.094 μ M, and YC1: 3782 μ M (Figure 3A). 17AAG at 0.025 μ M and 0.05 μ M did

Figure 2. siRNA knockdown of hypoxia-inducible factor-1α (HIF1α). HIF1α expression (A) and cell proliferation of ACCM cells (B) were blocked by HIF1α siRNA. HIF1α also significantly inhibited the invasion of ACC2 and ACCM cells (C and D).

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.

Figure 3. A: Inhibition of cell proliferation by hypoxia-inducible factor-1 α (HIF1 α) inhibitors and the concentration of HIF1 α inhibitor at which 50% of cell growth was inhibited compared to the control (IC_{50}). B: Echinomycin inhibited growth of ACC2 cells. C: Invasion of ACC2 and ACCM cells was significantly blocked by 0.0008 μ M echinomycin and 0.05 μ M 17-N-allylamino-17-demethoxygeldanamycin (17AAG).

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

Figure 4. Lung metastasis model and inhibition by hypoxia-inducible factor-1 α (HIF1 α) inhibitors. Multiple metastatic nodules are visible on gross (A) and histological (B) examination (staining, $\times 100$). C: Animal study protocol. 17-N-Allylamino-17-demethoxygeldanamycin (17AAG) reduced the number of metastatic foci (D). W: Weeks.

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. HIF1 α is a potential prognostic

biomarker in proteomic assessments of breast and prostate cancer (13, 14). Schrijvers *et al.* reported that HIF1 α 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 HIF1 α and VEGF expression was significantly associated with tumors located in the lower lip and larynx (24).

In addition, the expression of HIF1 α is increased in papillary thyroid carcinoma (PTC), the most common type of head and neck cancer. Scarpino *et al.* reported that the levels of HIF1 α 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 HIF1 α in patients with ACC. Costa *et al.* reported that HIF1 α expression is a common finding in

ACC (26). Hara *et al.* reported that hypoxia activates the HGF/c-Met system *via* HIF1 α 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 HIF1 α in ACC *in vitro* and in *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 HIF1 α siRNA and 17AAG blocked the invasiveness of BHP10-3SC (a tumorigenic subclone from human PTC cell lines BHP10-3) by inhibition of HIF1 α 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 HIF1 α and HIF1 β 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. Vlamincik *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 HIF1 α 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 HIF1 α expression in the cell lines tested, and that HIF1 α is important in metastatic tumor growth in ACC. HIF1 α 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 HIF1 α 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

- 1 Kim KH, Sung MW, Chung PS, Rhee CS, Park CI and Kim WH: Adenoid cystic carcinoma of the head and neck. Arch Otolaryngol Head Neck Surg 120: 721-726, 1994.
- 2 Sung MW, Kim KH, Kim JW, Min YG, Seong WJ, Roh JL, Lee SJ, Kwon TK and Park SW: Clinicopathologic predictors and impact of distant metastasis from adenoid cystic carcinoma of the head and neck. Arch Otolaryngol Head Neck Surg 129: 1193-1197, 2003.
- 3 Spiro RH: Distant metastasis in adenoid cystic carcinoma of salivary origin. Am J Surg 174: 495-498, 1997.
- 4 Carmeliet P and Jain RK: Angiogenesis in cancer and other diseases. Nature 407: 249-257, 2000.
- 5 Shen J, Hughes C, Chao C, Cai J, Bartels C, Gessner T and Subjeck J: Coinduction of glucose-regulated proteins and doxorubicin resistance in Chinese hamster cells. Proc Natl Acad Sci USA 84: 3278-3282, 1987.
- 6 Hockel M, Schlenger K, Aral B, Mitze M, Schaffer U and Vaupel P: Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. Cancer Res 56: 4509-4515, 1996.
- 7 Hockel M and Vaupel P: Tumor hypoxia: definitions and current clinical, biologic and molecular aspects. J Natl Cancer Inst 93: 266-276, 2001.
- 8 Kizaka-Kondoh S, Inoue M, Harada H and Hiraoka M: Tumor hypoxia: a target for selective cancer therapy. Cancer Sci 94: 1021-1028, 2003.

- 9 Semenza GL: Targeting HIF1 for cancer therapy. *Nat Rev Cancer* 3: 721-732, 2003.
- 10 Semenza GL: Regulation of mammalian O₂ homeostasis by hypoxia-inducible factor 1. *Annu Rev Cell Dev Biol* 15: 551-578, 1999.
- 11 Bunn HF and Poyton RO: Oxygen sensing and molecular adaptation to hypoxia. *Physiol Rev* 76: 839-885, 1996.
- 12 Wang GL, Jiang BH, Rue EA and Semenza GL: Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O₂ tension. *Proc Natl Acad Sci USA* 92: 5510-5514, 1995.
- 13 Zhong H, Semenza GL, Simons JW and De Marzo AM: Up-regulation of hypoxia-inducible factor 1alpha is an early event in prostate carcinogenesis. *Cancer Detect Prev* 28: 88-93, 2004.
- 14 Dales JP, Garcia S, Meunier-Carpentier S andrac-Meyer L, Haddad O, Lavaut MN, Allasia C, Bonnier P and Charpin C: Overexpression of hypoxia-inducible factor HIF1alpha predicts early relapse in breast cancer: retrospective study in a series of 745 patients. *Int J Cancer* 116: 734-739, 2005.
- 15 Mimnaugh EG, Chavany C and Neckers L: Polyubiquitination and proteasomal degradation of the p185c-erbB-2 receptor protein-tyrosine kinase induced by geldanamycin. *J Biol Chem* 271: 22796-22801, 1996.
- 16 Guan XF, Qiu WL, He RG and Zhou XJ: Selection of adenoid cystic carcinoma cell clone highly metastatic to the lung: an experimental study. *Int J Oral Maxillofac Surg* 26: 116-119, 1997.
- 17 Vaupel P: The role of hypoxia-induced factors in tumor progression. *Oncologist* 9(Suppl 5): 10-17, 2004.
- 18 Vaupel P, Kelleher DK and Hockel M: Oxygen status of malignant tumors: pathogenesis of hypoxia and significance for tumor therapy. *Semin Oncol* 28: 29-35, 2001.
- 19 Gray LH: Radiobiologic basis of oxygen as a modifying factor in radiation therapy. *Am J Roentgenol Radium Ther Nucl Med* 85: 803-815, 1961.
- 20 Amellem O and Pettersen EO: Cell inactivation and cell cycle inhibition as induced by extreme hypoxia: the possible role of cell-cycle arrest as a protection against hypoxia-induced lethal damage. *Cell Prolif* 24: 127-141, 1991.
- 21 Griguer CE, Oliva CR, Gobin E, Marcotelles P, Benos DJ, Lancaster JR, Jr. and Gillespie GY: CD133 is a marker of bioenergetic stress in human glioma. *PLoS One* 3: e3655, 2008.
- 22 Kim H, Peng G, Hicks JM, Weiss HL, Van Meir EG, Brenner MK and Yotnda P: Engineering human tumor-specific cytotoxic T-cells to function in a hypoxic environment. *Mol Ther* 16: 599-606, 2008.
- 23 Schrijvers ML, van der Laan BF, de Bock GH, Pattje WJ, Mastik MF, Menkema L, Langendijk JA, Kluin PM, Schuurin E and van der Wal JE: Overexpression of intrinsic hypoxia markers HIF1alpha and CA-IX predict for local recurrence in stage T1-T2 glottic laryngeal carcinoma treated with radiotherapy. *Int J Radiat Oncol Biol Phys* 72: 161-169, 2008.
- 24 Kyzas PA, Stefanou D, Batistatou A and Agnantis NJ: Hypoxia-induced tumor angiogenic pathway in head and neck cancer: an *in vivo* study. *Cancer Lett* 225: 297-304, 2005.
- 25 Scarpino S, Cancellario d'Alena F, Di Napoli A, Pasquini A, Marzullo A and Ruco LP: Increased expression of Met protein is associated with up-regulation of hypoxia inducible factor-1 (HIF1) in tumour cells in papillary carcinoma of the thyroid. *J Pathol* 202: 352-358, 2004.
- 26 Costa AF, Tasso MG, Mariano FV, Soares AB, Chone CT, Crespo AN, Fresno MF, Llorente JL, Suarez C, de Araujo VC, Hermesen M, and Altemani A: Levels and patterns of expression of hypoxia-inducible factor-1alpha, vascular endothelial growth factor, glucose transporter-1 and CD105 in adenoid cystic carcinomas with high-grade transformation. *Histopathology* 60: 816-825, 2012.
- 27 Hara S, Nakashiro K, Klosek SK, Ishikawa T, Shintani S, and Hamakawa H: Hypoxia enhances c-Met/HGF receptor expression and signaling by activating HIF-1alpha in human salivary gland cancer cells. *Oral Oncol* 42: 593-598, 2006.
- 28 Ahn SH, Choi JY, Kim DW, Lee DY, Jeon EH, Jeong WJ and Paik JH: Targeting HIF1alpha perioperatively increased post-surgery survival in a tongue cancer animal model. *Ann Surg Oncol* 22: 3041-3048, 2015.
- 29 Mo JH, Choi IJ, Jeong WJ, Jeon EH and Ahn SH: HIF1alpha and HSP90: target molecules selected from a tumorigenic papillary thyroid carcinoma cell line. *Cancer Sci* 103: 464-471, 2012.
- 30 Formica JV and Waring MJ: Effect of phosphate and amino acids on echinomycin biosynthesis by *Streptomyces echinatus*. *Antimicrob Agents Chemother* 24: 735-741, 1983.
- 31 Van Dyke MM and Dervan PB: Echinomycin binding sites on DNA. *Science* 225: 1122-1127, 1984.
- 32 Kong D, Park EJ, Stephen AG, Calvani M, Cardellina JH, Monks A, Fisher RJ, Shoemaker RH, and Melillo G: Echinomycin, a small-molecule inhibitor of hypoxia-inducible factor-1 DNA-binding activity. *Cancer Res* 65: 9047-9055, 2005.
- 33 Vlaminck B, Toffoli S, Ghislain B, Demazy C, Raes M and Michiels C: Dual effect of echinomycin on hypoxia-inducible factor-1 activity under normoxic and hypoxic conditions. *FEBS J* 274: 5533-5542, 2007.

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