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A hypoxia-inducible factor (HIF)-3α splicing variant, HIF-3α4 impairs angiogenesis in hypervascular malignant meningiomas with epigenetically silenced HIF-3α4

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ABSTRACT

Hypoxia inducible factor is a dominant regulator of adaptive cellular responses to hypoxia and controls the expression of a large number of genes regulating angiogenesis as well as metabolism, cell survival, apoptosis, and other cellular functions in an oxygen level-dependent manner. When a neoplasm is able to induce angiogenesis, tumor progression occurs more rapidly because of the nutrients provided by the neovasculature. Meningioma is one of the most hypervascular brain tumors, making anti-angiogenic therapy an attractive novel therapy for these tumors. HIF-3α has been conventionally regarded as a dominant-negative regulator of HIF-1α, and although alternative HIF-3α splicing variants are extensively reported, their specific functions have not yet been determined. In this study, we found that the transcription of HIF-3α4 was silenced by the promoter DNA methylation in meningiomas, and inducible HIF-3α4 impaired angiogenesis, proliferation, and metabolism/oxidation in hypervascular meningiomas. Thus, HIF-3α4 could be a potential molecular target in meningiomas.

1. Introduction

Brain tumors are classified into grades I–IV by the World Health Organization (WHO) according to histological features, with grade I being the most benign and grade IV being the most severe. Meningiomas are frequent neoplasms accounting for approximately 25% of all intracranial tumors [11]. They are mainly characterized by a benign histology and an indolent clinical course, and most are pathologically diagnosed as WHO grade I and curable by surgical resection. However, grade II or III meningiomas are occasionally encountered. Even more troublesome, some populations of meningiomas with benign pathological findings (WHO grade I) pursue a malignant course [12–14]. Our group has previously reported the use of whole genome methylation analysis to predict which tumors will undergo this malignant clinical course, and we were able to predict the outcome in meningioma patients on the basis of the methylation status of 5 hub genes, including HIF-3α [15].

When a neoplasm is able to induce angiogenesis, tumor progression occurs more rapidly because of the nutrients provided by the neovasculature [1–3]. Meningioma is one of the most hypervascular tumors, making anti-angiogenic therapy an attractive novel therapy for these tumors [16]. This therapy targets angiogenetic factors, including vascular endothelial growth factor (VEGF) and platelet-derived growth factor, both of which are regulated by hypoxia inducible factor (HIF) [4,17,18].

HIF is a dominant regulator of adaptive cellular responses to hypoxia and controls the expression of a large number of genes regulating angiogenesis as well as metabolism, cell survival, apoptosis, and other cellular functions in an oxygen level-dependent manner. HIF forms a dimer consisting of an unstable α subunit (HIF-α) and a stable β subunit (HIF-β). This dimer binds to specific sequences termed hypoxia response elements (HRE) in the promoter region of HIF target genes such as VEGF. There are 3 principal isoforms of the HIF-α subunit (HIF-1α, 2α, 3α). HIF-1α and HIF-2α share a similar domain architecture and undergo similar proteolytic regulation [4–6,10,17,19,20]. HIF-3α, on the other hand, has been

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conventionally regarded as a dominant-negative regulator of HIF-1α [4–10], and although alternative HIF-3α splicing variants are extensively reported, their specific functions have not yet been determined [5,7,8,10,21]. HIF-3α4, one of the HIF-3α splicing variants, is similar to mouse inhibitory Per/Arnt/Sim domain protein (IPAS). IPAS expression in hepatoma cells selectively impairs tumor vascular density, and inhibition of IPAS expression induces vascular growth in the cornea of mice [9].

In this study, we constructed a meningioma cell line stably expressing HIF-3α4 in order to elucidate the function of HIF-3α4 in hypervascular malignant meningioma. We addressed multiple novel functions of HIF-3α4 in hypervascular meningiomas; angiogenesis, proliferation, and metabolism/oxidation. HIF-3α4 could be a potential molecular target in meningiomas.

2. Materials and methods

2.1. Cell lines

The human meningioma cell lines IOMM-Lee and HKBMM were kindly provided by Drs. Anita Lai (University of California at San Francisco, CA) and Shinichi Miyatake (Osaka Medical University, Osaka, Japan), respectively. All cell lines were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cell lines were grown at 37 °C in a humidified atmosphere of 5% CO_2 under normoxic (20% O_2) or hypoxic (1% O_2) conditions.

2.2. Genetically engineered meningioma cells

Using the following protocol, we designed IOMM-Lee or HKBMM meningioma cell lines stably expressing green fluorescence protein (GFP) or GFP-tagged HIF-3α4, designated IO-GFP, IO-HIF3α4, HK-GFP, and HK-HIF3α4, respectively. pQCXIP-HIF3α4-GFP plasmid was prepared by the following protocol: HIF-3α transcript variant 3, isoform c complete cDNA (accession No. BC080551) was obtained from GeneCopoeia (Rockville, MD). This cDNA was amplified using the forward primer 5′-CTAGATGAATTCATGGCGCTGGGGCTGCAGCG-3′, including an *Eco*RI site and the reverse primer 5′-CTAGATCGCGGCCGCTCAGCTCAGCAAGGTGTGGATGC-3′, including a *Not*I site. Oligonucleotides were obtained from Greiner Japan (Tokyo, Japan). After amplification, the products were purified by a QIAquick® Spin kit (QIAGEN, Hilden, Germany) and sequenced on an automated DNA sequencer (ABI PRISM 310, Applied Biosystems, Foster City, CA). Then, the products were digested by *Eco*RI and *Not*I (Takara Bio, Otsu, Japan), and the DNA was inserted to pQCXIP retrovirus vector (Clontech, Mountain View, CA) that had previously been altered to contain the GFP gene by using the DNA Ligation kit (Takara Bio, Otsu, Japan). These plasmids were co-transfected with pVSV-G (Clontech) into the retroviral packaging cell line 293T using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) to produce retrovirus. At 24 h post-transfection with pQCXIP-HIF3α4-GFP and pVSV-G, the medium was changed to DMEM. After another 24 h, the supernatant containing viral particles was collected and filtered through a 0.45-μm-pore PVDF membrane filter (Millex®-HV Syringe Driven Filter Unit; Millipore, Bradford, MA). IOMM-Lee or HKBMM meningioma cells were incubated for 48 h with the viral supernatant plus 4 μg/mL polybrene infection/transfection reagent (Millipore). Cells were then treated with 10 μg/mL puromycin (Sigma–Aldrich, St. Louis, MO) for 1 week to select the cells stably expressing HIF-3α4 and GFP.

2.3. Demethylation treatment

Cells (1×10^5 cells) were seeded in 6-well plate and incubated for 24 h, then treated with 5-aza-2′-deoxycytidine at the final concentration of 0, 1 or 5 μM. After the first administration, the same dose agent was added four times in total every 12 h. At 12 h after the fourth administration, medium was changed, and cells then were collected and RNA was extracted.

2.4. RNA extraction, reverse transcriptase PCR, and quantitative real-time PCR

RNA was extracted from IOMM-Lee or HKBMM cell line using Trizol® (Invitrogen, Carlsbad, CA). The following gene-specific oligonucleotide primers were obtained from Greiner Japan: HIF-1α forward 5′-GAGCTTGCTCATCAGTTGCC-3′ and reverse 5′-CTGTA CTGTCCTGTGGTGAC-3′, and HIF-3α4 forward 5′-CCCAGAGCTCAGAGGACGAG-3′ and reverse 5′-CCCAACACACCAGGCTGAGA-3′. First-strand cDNA was synthesized by Transcriptor First Strand cDNA synthesis kit (Roche, Indianapolis, IN) according to the manufacturer's instructions. The resulting products were analyzed on a 2% agarose gel stained with ethidium bromide. Quantitative real-time PCR (qPCR) was performed using the LightCycler 480 system (Roche Applied Science, Mannheim, Germany) and the Light Cycler 480 SYBR Green I Master (Roche).

2.5. Antibodies

Anti-HIF-1α antibody and anti-HIF-3α antibody were obtained from Novus Biologicals (Littleton, CO). Anti-GFP antibody was from MBL (Nagoya, Japan). Anti-β-actin antibody was from Sigma–Aldrich.

2.6. Protein extracts and western blot analysis

Meningioma cell lysates were prepared by lysing cells in 200 μL of 2× lysis buffer (20% [v/v] glycerol, 13.5% [v/v] 1 M Tris–HCl, 40% [v/v] 10% SDS, 4.0 ng bromophenol blue, 10% [v/v] mercaptoethanol, 16.5% [v/v] water). Lysates (10 μL) were loaded into 10% Mini-PROTEAN TGX Gels (BIO-RAD, Hercules, CA). Separated proteins were electrotransferred onto PVDF membranes (Hybond-P, GE Healthcare, Buckinghamshire, United Kingdom); then, western blots were performed using the antibodies listed above. Bands were detected using ECL Western Blotting Detection Reagents (GE Healthcare).

2.7. Protein interaction assay

Meningioma cells were seeded in 10-cm cell culture dishes and lysed in TNE buffer (150 mM NaCl, 35 mM Tris–HCl, 1% NP-40, 1 mM EDTA, 10 μg/mL aprotinin) at 4 °C. The lysates were immunoprecipitated with the anti-GFP antibody bound to Pierce® Protein G Agarose (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions. The immunoprecipitated samples were analyzed by western blotting as described above.

2.8. Wound scratch assay

Meningioma cells were seeded in 10-cm tissue culture dishes at a concentration of 30×10^4 cells/mL and cultured under normoxia or hypoxia. Linear wounds were generated in the monolayer with the tips of a sterile 200-μL plastic pipette. Cellular debris was removed by washing with phosphate buffered saline (PBS). PBS was removed, and DMEM supplemented with 2% [v/v] FBS and 1% [v/v] penicillin–streptomycin was added to the culture dishes. Every 6 h, wounds were photographed at 20 scratched points per dish and each wound area was estimated using Image J software (Rasband, W.S., Image J, National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij/, 1997–2009).