Endolymphatic sac tumors in patients with and without von Hippel–Lindau disease: the role of genetic mutation, von Hippel–Lindau protein, and hypoxia inducible factor-1α expression

RANDY L. JENSEN, M.D., PH.D., DAVID GILLESPIE, PH.D., PAUL HOUSE, M.D., LESTER LAYFIELD, M.D., AND CLOUGH SHELTON, M.D.

Departments of Neurosurgery, Surgery (Division of Otolaryngology), and Pathology, and Huntsman Cancer Institute, University of Utah, Salt Lake City; and Neurosurgical Section, Surgical Service, Department of Veterans Affairs, Salt Lake City Health Care System, Salt Lake City, Utah

Object. Endolymphatic sac (ELS) tumors are low-grade malignancies of the temporal bone that are associated with von Hippel–Lindau (VHL) disease but can also occur sporadically. The VHL gene product VHL protein is important in the regulation of hypoxia inducible factor (HIF)-1α, which controls expression of molecules that are important in angiogenesis and cell metabolism. In this study the authors examine the role of VHL and HIF-1 in ELS tumors.

Methods. The ELS tumors from three patients were examined using the following method: DNA from tumor tissue was isolated, amplified by polymerase chain reaction and the VHL gene sequence was compared with the known wild-type sequence. Loss of heterozygosity (LOH) studies were performed to confirm the sequencing data. Immunohistochemical evaluation for VHL, HIF-1α, vascular endothelial growth factor (VEGF), and carbonic anhydrase IX (CA IX) was performed. Snap-frozen tumor tissue was examined using Western blot and HIF-1 immunoassays for HIF-1α and VHL expression.

Two patients had sporadic ELS tumors and the other one suffered from VHL disease. Results of VHL gene sequencing were normal in the tissue derived from the sporadic ELS tumors. The ELS tumor, pheochromocytoma, and spinal hemangioblastoma were heterozygous for the same C-to-A transversion found in the germline carried by the patient with VHL disease. No LOH was detected in the tumor tissue obtained in the patient with VHL disease. Expression of HIF-1α, VEGF, and CA IX evaluated using immunohistochemical studies was elevated in the VHL-associated tumors. Nevertheless, Western blots and immunoassays for HIF-1α did not show elevated expression in these tumors.

Conclusions. The sporadic and VHL disease-associated ELS tumors in this study had normal VHL-mediated HIF-1α regulation. This is a result of normal VHL gene expression in the case of the sporadic ELS tumor. In the VHL-associated ELS tumor, this is due to one normal copy of the VHL gene and adequate VHL gene expression.

Key Words • endolymphatic sac • von Hippel–Lindau disease • hypoxia inducible factor

The ELS tumor is a low-grade adenocarcinoma of the temporal bone that is thought to originate from the vestibular ELS.11,24,29,46-48 Although ELS tumors can occur spontaneously, a significant number of them have been reported in the context of VHL disease.13,21,45,56,59,66 Mutations in the VHL gene have been reported in both the sporadic and VHL disease–associated ELS tumors.44,78,79 Nevertheless, because of the rarity of ELS tumors, few have been subjected to molecular genetic analysis.27

The pVHL has recently been shown to be important in the regulation of HIF-1α.10,36,39 Constitutive expression of HIF-1α has been detected in the cancer cells related to VHL disease.49,63,67,87 During conditions of low O2 tension (1–2% O2), degradation of HIF-1α is inhibited by O2-dependent hydroxylation of the protein that prevents interaction of HIF-1α with pVHL ubiquitin ligase complex (Fig. 1).10,36,39,50,70 The HIF-1α then binds to a DNA sequence known as HREs, and induces the transcription of a number of well-characterized genes that help cells “cope” with low O2 conditions; these include VEGF and almost every gene in the glycolytic pathway.67,80 In this study we examine the role of pVHL and HIF-1 in sporadic and VHL disease–associated ELS tumors.

Clinical Material and Methods

Patient Data

Three patients with ELS tumors were studied. Specimens were snap frozen in the operating room and also fixed in formalin and embedded in paraffin wax for the studies described later, detailed case histories and imaging studies are also described. Tumors obtained in the patient with known VHL disease included ELS tumor, pheochromocytoma, and a spinal hemangioblastoma. In both patients without known VHL disease, both the original and recurrent ELS
Role of VHL genetics, pVHL, and HIF-1α expression in ELS tumors

tumors were available for study (Table 1). Tumor specimens and case histories were used after permission was obtained from the patients according to an institutional review board–approved protocol.

Laser Capture Manual Microdissection

Paraffin-embedded tumors were deparaffinized in 100% xylene for 5 minutes, then rinsed in 70% ethanol for 5 minutes. Tumors were stained with hematoxylin for better visualization. Manual microdissection was performed with the aid of direct-light microscopic visualization by using a stereomicroscope as described previously. Briefly, areas of tissue sections containing predominantly tumor cells were selected and scraped with a modified Pasteur pipette coated with adhesive containing 568 g/L piccolyte and 437.5 g/L xylene (Biomedical Sciences, Inc., Bridgeport, NJ).

The LCM method provides a means to isolate a pure population of cells from heterogeneous tissue specimens. This method was used to confirm that sequencing results obtained using a standard manual microdissection procedure were not contaminated by nontumorous cells. The LCM was performed with the aid of direct microscopic observation by using an LCM system (Pixcell 2; Arcturus, Mountain View, CA) according to the manufacturer’s suggested method and that published by other groups. Briefly, areas of interest are selected by looking through the microscope. The transfer film is lowered to the tissue surface and the laser is pulsed, which activates the transfer film in the focal region of the laser beam. When the transfer film is lifted off, the selected cell(s) remain adherent to the film surface, and are placed directly into the digestion buffer described next.

Tumor VHL Sequencing

Tumor samples obtained by LCM were digested at 50°C overnight with 10 mM Tris-HCl (pH 8), 2 mM ethylenediamine tetraacetic acid, 0.2% Triton X-100, and 0.1 mg/ml proteinase K in a final volume of 10 μl. Manually microdissected tissue was digested overnight at 50°C in lysis buffer (Xtrana, Broomfield, CO) with 20 μg proteinase K. In either case, proteinase K was inactivated by 5 minutes of boiling. The DNA was extracted from the digested tissue samples by using a DNA extraction kit (Xtrana Xtra Amp series III; Xtrana). The DNA was bound to immobilized glass beads in 200-μl PCR tubes and washed according to the manufacturer’s protocol.

All three VHL exons were amplified by PCR in which intronic primers were used (Table 2) as described previously. The PCR amplification with isolated DNA was performed in two steps. First, a 50-μl PCR booster reaction was done according to the manufacturer’s instructions, using a 10-nM mix of VHL exon 1, 2, and 3 primer sets in buffer G (FailSafe; Epicentre, Madison, WI) with the following cycle parameters: 94°C, 5 minutes; 10 cycles of 94°C, 20 seconds; 58°C, 20 seconds; 72°C, 40 seconds, with a final extension of 72°C, 2 minutes.

The secondary PCR was done in 50 μl in which 5 μl of the booster reaction was used in template in the following conditions: VHL exon 1, FailSafe buffer G (Epicentre), 200 nM sense and antisense primers; VHL exons 2 and 3, 200 mM Tris HCl, 50 mM KCl, 1.5 mM MgCl2, 0.2 mM deoxyribonucleoside triphosphate, 200 nM sense and antisense primers, 2.5 μl DNA polymerase (Platinum Taq; Invitrogen, Carlsbad, CA). The following cycles for touchdown PCR were used for the secondary PCR of all three exons: 94°C, 5 minutes; eight cycles of 94°C, 15 seconds; 61°C, 15 seconds; 72°C, 20 seconds; then five cycles with an annealing temperature of 59°C, then 30 cycles of 94°C, 15 seconds; 54°C, 15 seconds; 72°C, 30 seconds, with a final extension for 5 minutes at 72°C. A PCR with DNA from microdissected tumor samples was performed, as described with the secondary PCR for isolated DNA. All PCRs were performed in a thermocycler (Geneamp 2400; Perkin-Elmer, Foster City, CA). The PCR products were purified before sequencing by using a PCR purification kit (Concert; Gibco BRL, Grand Island, NY). Sequencing of sense and antisense DNA was done at the University of Utah Sequencing Core Facility with primers used previously for PCR amplification (Table 2).

Loss of Heterozygosity Testing

Loss of heterozygosity analysis for chromosome 3p was performed using 3p microsatellite probes (D3S1304 and D3S1317) close to the VHL gene on somatic DNA extracted from tumor and leukocytes, as has been previously described.

Tumor DNA was extracted as described earlier. Leukocyte DNA was extracted using a DNA blood extraction kit (Qiagen, Inc., Valencia, CA). The LOH analysis was performed using paired normal-tumor microsatellite PCR. Forty nanograms of DNA was used for each PCR, with 20-μl reactions done in 96-well PCR trays. The primer D3S1304 (MD-10 Linkage Mapping Primer Set; Applied Biosystems, Foster City, CA) uses the dye HEX, which appears...
green on filter set D on the Applied Biosystems model 3100 capillary instrument. For this marker, the PCR conditions were as follows: 94°C for 5 minutes, then 94°C for 20 seconds; 55°C for 20 seconds; 72°C for 40 seconds for 35 cycles, then 72°C for 10 minutes. Primer D3S1317 (Integrated DNA Technology, Coralville, IA) uses the dye FAM, which appears blue on the same filter set. The PCR conditions for this marker were as follows (step-down program): 94°C for 5 minutes, then 94°C for 20 seconds; 64°C for 20 seconds; −1°C per cycle for eight cycles; 72°C for 40 seconds, then 94°C for 20 seconds; 56°C for 20 seconds; 72°C for 40 seconds for an additional 30 cycles, then 72°C for 10 minutes.

After the PCR, the samples were diluted with 140 μl of high-performance liquid chromatography water, then 0.5 μl of diluted PCR product for D3S1317 (FAM dye) and 1 μl of diluted PCR product for D3S1304 (HEX dye) were loaded on the model 3100 capillary instrument. The two PCR products were multiplexed because they were different sized on the model 3100 capillary instrument. For this marker, the PCR conditions were as follows: 94°C for 5 minutes, then 94°C for 20 seconds; 55°C for 20 seconds; 72°C for 40 seconds for 35 cycles; then 94°C for 20 seconds; 56°C for 20 seconds; 72°C for 40 seconds for an additional 30 cycles, then 72°C for 10 minutes.

Tumor tissue was snap frozen in the operating room and stored in liquid N₂. Tumor samples were chopped and 0.3 g of tissue was homogenized with a polytron in 3 ml of digestion buffer containing 10 mM N-2-hydroxyethylpipеризе-N’-2-ethanesulfonic acid (pH 7.6), 0.1 mM ethyleneglycoltetraacetic acid, 2 mM dithiothreitol, 0.4 mM phenylmethylsulfonate, 1 mM NaVO₃, 1X protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO), 100 mM NaF, and 10 mM Na₃PO₄. The homogenate was centrifuged at 850 G for 10 minutes at 4°C. The supernatant was transferred to a new tube and glycerol was added for a final concentration of 20%, then the cells were vortexed and centrifuged at 10,000 G for 10 minutes. The pellet was resuspended in 300 μl of lysis buffer (400 mM NaCl, 20 mM N-2-hydroxyethylpipеризе-N’-2-ethanesulfonic acid [pH 7.5], 10 mM NaF, 10 mM para-nitrophenylphosphate, 1 mM NaVO₃, 0.1 mM ethylenediaminetetraacetic acid, 10 μM Na₃PO₄, 10 mM β-glycerophosphate, and 20% glycerol) and shaken gently at 4°C for 30 minutes, then centrifuged at 15,000 G at 4°C for 30 minutes. Protein concentration was determined using the Bradford method (BioRad, Hercules, CA), and the supernatant was stored at −70°C.

**Western Blots**

Twenty-five micrograms of nuclear protein was separated on a 4 to 12% bis-Tris NuPAGE gel (Invitrogen) and transferred to a polyvinylidene difluoride membrane (Hybond-P; Amersham, Piscataway, NJ). The membrane was blocked overnight in 5% milk/Tris-buffered saline/1% Tween 20 and probed with mouse immunoglobulin G anti-human HIF-1α mAb HIF1α-67 (dilution 1:1000; Novus Biologicals, Littleton, CO), anti-VEGF Ab-1 polyclonal antibody (1:50 dilution; Calbiochem, Cambridge, MA), mouse mAb anti-human CA IX M75 (1:50; Dako), and mouse mAb anti-human VHL IG33 (1:200; Neomarker, Fremont, CA). Negative control evaluations were performed by replacing the primary antibody with nonimmune serum. Sections were lightly counterstained with toluidine blue and dehydrated in a graded series of alcohols and xylene; coverslips were applied with Permount mounting media (Fisher Scientific, Pittsburgh, PA).

**Protein Isolation From Tumors**

**TABLE 1**

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age (yrs)</th>
<th>Sex</th>
<th>VHL Disease</th>
<th>Tumor Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>32</td>
<td>F</td>
<td>yes</td>
<td>ELS, pheochromocytoma, spinal hemangioblastoma</td>
</tr>
<tr>
<td>2</td>
<td>45</td>
<td>F</td>
<td>no</td>
<td>ELS, recurrent tumor</td>
</tr>
<tr>
<td>3</td>
<td>62</td>
<td>F</td>
<td>no</td>
<td>ELS, recurrent tumor</td>
</tr>
</tbody>
</table>

**Light Microscopy and Immunohistochemical Studies**

Formalin-fixed, paraffin-embedded surgical specimens were sectioned at a thickness of 6 μm, warmed to 60°C for 20 minutes, deparaffinized in xylene, and hydrated in a graded series of ethanol/H₂O. Routine sections were stained with H & E for histological diagnosis.

The HIF-1α immunohistochemical examination was performed using the catalyzed signal amplification system (Dako, Carpinteria, CA). We performed VEGF, VHL, and CA IX immunohistochemical studies by using the Vectastain avidin–biotin complex kit (Vector Laboratories, Burlingame, CA). Primary antibodies used were mouse mAb anti-human HIF-1α H1α67 (dilution 1:1000; Novus Biologicals, Littleton, CO), anti-VEGF Ab-1 polyclonal antibody (1:50 dilution; Calbiochem, Cambridge, MA), mouse mAb anti-human CA IX M75 (1:50; Dako), and mouse mAb anti-human VHL IG33 (1:200; Neomarker, Fremont, CA). Negative control evaluations were performed by replacing the primary antibody with nonimmune serum. Sections were lightly counterstained with toluidine blue and dehydrated in a graded series of alcohols and xylene; coverslips were applied with Permount mounting media (Fisher Scientific, Pittsburgh, PA).

**Protein Isolation From Tumors**

Tumor tissue was snap frozen in the operating room and stored in liquid N₂. Tumor samples were chopped and 0.3 g of tissue was homogenized with a polytron in 3 ml of digestion buffer containing 10 mM N-2-hydroxyethylpipеризе-N’-2-ethanesulfonic acid (pH 7.6), 0.1 mM ethyleneglycoltetraacetic acid, 2 mM dithiothreitol, 0.4 mM phenylmethylsulfonate, 1 mM NaVO₃, 1X protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO), 100 mM NaF, and 10 mM Na₃PO₄. The homogenate was centrifuged at 850 G for 10 minutes at 4°C. The supernatant was transferred to a new tube and glycerol was added for a final concentration of 20%, then the cells were vortexed and centrifuged at 10,000 G for 10 minutes. The pellet was resuspended in 300 μl of lysis buffer (400 mM NaCl, 20 mM N-2-hydroxyethylpipеризе-N’-2-ethanesulfonic acid [pH 7.5], 10 mM NaF, 10 mM para-nitrophenylphosphate, 1 mM NaVO₃, 0.1 mM ethylenediaminetetraacetic acid, 10 μM Na₃PO₄, 10 mM β-glycerophosphate, and 20% glycerol) and shaken gently at 4°C for 30 minutes, then centrifuged at 15,000 G at 4°C for 30 minutes. Protein concentration was determined using the Bradford method (BioRad, Hercules, CA), and the supernatant was stored at −70°C.

**Western Blots**

Twenty-five micrograms of nuclear protein was separated on a 4 to 12% bis-Tris NuPAGE gel (Invitrogen) and transferred to a polyvinylidene difluoride membrane (Hybond-P; Amersham, Piscataway, NJ). The membrane was blocked overnight in 5% milk/Tris-buffered saline/1% Tween 20 and probed with mouse immunoglobulin G anti-human HIF-1α mAb H72320 (1:2000; Transduction Laboratories, Lexington, KY), mouse anti-VHL (1:1000; BD Biosciences, San Diego, CA), and mouse immunoglobulin

**TABLE 2**

<table>
<thead>
<tr>
<th>Set</th>
<th>Exon</th>
<th>Position</th>
<th>Sequence of Primers</th>
<th>Authors &amp; Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>forward</td>
<td>5'-GAAATACAGTAACGAGTTGAGCTTAGC</td>
<td>Clifford, et al., 1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>reverse</td>
<td>5'-GACCGTGCTATCGTCCTGC</td>
<td>Bradley, et al., 1999</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>forward</td>
<td>5'-GCGGAGGAGGGACGGCGTT</td>
<td>Bender, et al., 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>reverse</td>
<td>5'-CGACTCCCTCCCCCGCGGT</td>
<td>Shuin, et al., 1994</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>forward</td>
<td>5'-GTGGCTCTTAAACACCTTTGC</td>
<td>Shuin, et al., 1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td>reverse</td>
<td>5'-CCTGACTCTTAAACACCTTTATC</td>
<td>Shuin, et al., 1994</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>forward</td>
<td>5'-TTCCTGTATGCAGAGCTTAG</td>
<td>Shuin, et al., 1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td>reverse</td>
<td>5'-AGCTGAGAGATGACGTGAAGT</td>
<td>Shuin, et al., 1994</td>
</tr>
</tbody>
</table>
Biosciences, San Diego, CA), and mouse immunoglobulin G antiactin mAb 1501 (1:4000; Chemicon International, Inc., Temecula, CA). The Western blots were visualized using enhanced chemiluminescence reagents (Amersham) and X-OMAT film (Eastman Kodak, Rochester, NY). A positive control for HIF-1α is provided with the antibody (HIF-1α protein isolated from CoCl2-treated COS-7 cells).

**Immunohistochemical Studies**

We examined all tumors resected in the patients in this study for somatic VHL mutations by direct sequencing of the coding region. Tumor cells were selectively studied using LCM. Sequencing of DNA extracted from ELS tumors obtained in the two patients without VHL disease revealed a normal wild-type sequence of the VHL gene (Genbank accession number L15409); this included both the original and recurrent ELS tumor obtained in one patient and the single ELS tumor in the other. The ELS tumor, pheochromocytoma, and spinal hemangioblastoma obtained in the patient with VHL disease all displayed the same C-to-A transversion at position -1 on the exon 3 splice acceptor sequence in the VHL gene (Fig. 3). A similar germline mutation has also been found in the patient, her father, and two sons. Interestingly, all tissue studied appeared to be heterozygous for the wild-type gene and this mutation (equal-sized peaks on the sequence histograms, Fig. 3A).

**Microsatellite LOH Analysis**

Microsatellite LOH analysis was conducted to compare DNA from leukocytes obtained in the patient with known VHL disease and the tumor tissue obtained in that patient; analysis was conducted using 3p microsatellite probes. Neither the ELS tumor, spinal hemangioblastoma, nor pheochromocytoma show LOH with both probes on 3p (Fig. 3B).

**Expression of HIF-1, VEGF, CA IX, and VHL on Immunohistochemical Studies**

Microscopically, ELS tumors showed proliferation of cuboidal tumor cells forming papillotubular structures and occasional cystic spaces (Fig. 4); mitotic figures were not found. The HIF-1α, VEGF, and CA IX expression functions were increased in the tumors obtained in the patient with known VHL disease and a mutation in the gene for VHL. The expression of these proteins is comparatively low in tumors obtained in patients without VHL disease. Conversely, expression of pVHL was low in the tumor obtained in the patient with VHL disease and normal expression in the spontaneous ELS tumors.

**Expression of HIF-1 and VHL on Western Blots**

Expression of HIF-1α was low in all tumors but slightly
increased in the spinal hemangioblastoma obtained in the patient with VHL disease and the ELS tumor obtained in the patient without VHL disease (Fig. 5). The HIF-1α extracted from CoCl-treated COS-7 cells served as a positive control for HIF-1α. Unexpectedly, pVHL expression was decreased in the ELS tumor obtained in the patient without VHL disease. We repeated the test a number of times and this result was confirmed, even with increased amounts of total protein from this tumor added for Western blot analysis (see actin band, Fig. 5). Expression of VHL in the spinal hemangioblastoma and ELS tumor obtained in the patient with known VHL disease has two bands, presumably from the wild-type and mutant proteins. This is consistent with the 6- to 7-kD predicted size change in the protein as a result of loss of exon 3 due to the splice acceptor mutation found in the VHL gene sequence of these tumors. The HIF-1 control was commercially supplied with the antibody and consisted of immunoprecipitated and concentrated HIF-1 protein, so the band seen in the VHL Western blot presumably represents nonspecific binding activity.

**Expression of HIF on Immunoassays**

Expression of HIF-1α measured by immunoassay was low for all of the ELS tumors and spinal hemangioblastomas compared with two human GBMs, a metastatic melanoma lesion, and the human GBM cell line U251 under both normoxic and hypoxic conditions (Fig. 6). Because of limited tumor tissue availability, multiple measurements were not possible in all cases, so comparisons between different ELS tumors are difficult to perform with statistical certainty. Similar to the Western blot analysis, however, HIF-1α expression was highest in the spinal hemangioblastoma obtained in the patient with VHL disease (VHL−) and one of the ELS tumors obtained in one of the patients without VHL (VHL+). The HIF-1α expression in the other patient without VHL disease was similar to the ELS tumor HIF-1α expression in the patient with VHL disease.

**Discussion**

**Endolymphatic Sac Tumors**

Adenoid tumors of the temporal bone were first reported in 1898 and have since included many names and classifications. ELS tumor emerged as a distinct entity in the late 1980s. The presence of a polypoid external auditory canal mass, facial paralysis, and evidence of a de-
Role of VHL genetics, pVHL, and HIF-1α expression in ELS tumors

Constructive mass arising on the posterior fossa surface of the temporal bone are common physical and radiographic findings in ELS tumors. Nevertheless, reports of ELS tumors mimicking other lesions have been made. Histologically, an ELS tumor is characterized by an infiltrative, poorly circumscribed proliferation of cuboidal cells forming a papillotubular pattern and occasional colloid-filled cysts and is reminiscent of thyroid papillary carcinoma. Mitotic figures are rarely present, necrosis is absent, and pleomorphism is minimal. Although ELS tumors are extremely rare in the general population, recently they have been associated with VHL disease.

Genetic Factors and VHL Disease

In addition to ELS tumors, patients with VHL disease are predisposed to renal cell carcinoma, cysts in the kidney or pancreas, various hemangioblastomas, and pheochromocytomas. The VHL tumor suppressor gene codes for a 213–amino acid protein (pVHL), which is involved in the regulation of angiogenesis, extracellular matrix formation, and which plays a role in the cell cycle. Transmission of VHL disease occurs in an autosomal–dominant manner. The VHL gene resides on chromosome 3p25-26 and is composed of three exons; mutations in any exon can result in VHL disease.

Individuals with VHL disease have typically inherited a
defective \textit{VHL} allele from one of their parents. Development of cysts or tumors is linked to the somatic inactivation or loss of the remaining wild-type allele. Genotype–phenotype correlations in \textit{VHL} disease demonstrate various patterns.\textsuperscript{18,61} For example, patients with \textit{VHL} missense mutations, especially in exon 3, have a high risk of pheochromocytoma (Type 2 \textit{VHL} disease).\textsuperscript{6,11,62,86} Our patient with \textit{VHL} disease appears to suffer from this variant of \textit{VHL} disease. It has been hypothesized that some retention of p\textit{VHL} function is necessary in the development of pheochromocytoma, possibly mediated as a dominant-negative effect of mutated p\textit{VHL}.\textsuperscript{41,72} Unfortunately it is not always this simple; there are reports of two distinct phenotypes caused by two different missense mutations in the same codon of the \textit{VHL} gene.\textsuperscript{3}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure6.png}
\caption{Bar graph showing HIF-1\textalpha expression measured by immunoassay. The HIF-1\textalpha is low for the ELS tumors and spinal hemangioblastoma compared with two human GBMs, a metastatic melanoma lesion (Met Mel), and the human GBM cell line U251 under both normoxic (Norm) and hypoxic (Hypo) conditions. Because of limited tumor tissue availability, multiple measurements were not possible in all cases and comparisons between different ELS tumors are difficult to do with statistical certainty. Similar to the Western blot analysis, however, HIF-1\textalpha expression is highest in the spinal hemangioblastoma obtained in the patient with \textit{VHL} disease and in one of the ELS tumors obtained in one of the patients without \textit{VHL} disease. In the other patient without \textit{VHL} disease, the HIF-1\textalpha expression was similar to that in the ELS tumor obtained in the patient with \textit{VHL} disease.}
\end{figure}

\textit{Mutations of the \textit{VHL} Gene in ELS Tumors}

Because of the rarity of ELS tumors, only a few have been subjected to molecular genetic analysis.\textsuperscript{27} A germline mutation of the \textit{VHL} gene and somatic loss of the wild-type allele were shown in ELS tumors obtained in patients with \textit{VHL} disease.\textsuperscript{44,76,78} Somatic \textit{VHL} gene mutations were detected in most but not all of the few sporadic ELS tumors studied.\textsuperscript{27,79}

We know that \textit{VHL} disease conforms to the Knudson two-hit model of carcinogenesis; namely, the development of \textit{VHL} disease–associated neoplasms is linked to the inactivation or loss of both the maternal and paternal \textit{VHL} alleles. Renal cell carcinoma in \textit{VHL} disease appears to require loss of the wild-type allele.\textsuperscript{24,53,71,83,88} In fact, restoration of p\textit{VHL} function in \textit{VHL}\textsuperscript{-/-} renal carcinoma cells is sufficient to suppress their ability to form tumors in nude mice.\textsuperscript{5,12,25,52,64} Loss of both copies of the \textit{VHL} gene also appears to be necessary for the development of central nervous system hemangioblastomas in patients with \textit{VHL} disease; however, this did not appear to be the case in the patient in our study.\textsuperscript{9,17,22,25,30,81} Our results could be explained by the limited amount of tissue available for study because of the small size of the tumor and damage from coagulation during resection.

Pheochromocytomas, on the other hand, do not always follow the two-hit model in either patients with \textit{VHL} disease or in sporadic cases.\textsuperscript{32,41} Indeed, many cases of seemingly sporadic pheochromocytoma have been linked, on further analysis, to other non-\textit{VHL} genetic alterations.\textsuperscript{2,32,60,62} It is also possible that the wild-type gene is inactivated by other mechanisms such as DNA hypermethylation, microdeletion, unrecognized point mutation, or a mechanism that has not yet been discovered.\textsuperscript{26,30,37} In our study, neither the pheochromocytoma nor ELS tumors appear to have \textit{VHL} genetic mutations.
Role of pVHL genetics, pVHL, and HIF-1α expression in ELS tumors

Presence of pVHL and HIF-1 Control

The pVHL has recently been shown to be important in the regulation of HIF-1α,4,5,36,39 HIF-1α is a transcription factor composed of two subunits: HIF-1αβ, also known as aryl hydrocarbon receptor nuclear translocator, and HIF-1α. At the messenger RNA level, HIF-1α and HIF-1β are both constitutively expressed. Under normoxic conditions (20% O2) HIF-1α is rapidly broken down by proteasomal degradation.7,10,38,40,74 This is mediated by interaction with the E3 ubiquitin ligase complex composed of pVHL, elongins B and C, Rbx1, and culin-2, which acts as the recognition component for polyubiquitination of proteins to “tag” them for proteasomal degradation (Fig. 1).45,65,72 The interaction of pVHL and HIF-1α is governed by the enzymatic hydroxylation of conserved proline within the pVHL-binding domain on the HIF-1α subunit.41,34,45

During low O2 tension conditions (1–2% O2), degradation of HIF-1α is inhibited.4,5,36,39,50,70 The HIF-1α translocates to the nucleus, binds HREs, and induces the transcription of a number of well-characterized genes that help cells cope with low O2 conditions; these include VEGF and almost every gene in the glycolytic pathway (Fig. 1).67,80 Mutations in any part of this system result in loss of hypoxia-mediated HIF-1α regulation, that is to say that HIF-1α is always “on” as an active transcription factor.43,72,75 Studies using HIF-1α variants that were engineered to escape recognition by pVHL could override the tumor suppressor activities of pVHL both in vivo and in vitro.48,57

Activation of HIF-1α appears to be an early event in the development of renal cell carcinoma.55 Constitutive expression of HIF-1α has been detected in the cancer cells related to VHL disease.41,63,67,77 After restoration of wild-type pVHL into these cells, O2-dependent expression of HIF-1α and HIF transcriptional activity is restored.25,34,48,58,68,73 Impaired ability to degrade the HIF-1α subunit appears to be required for development of hemangioblastoma and susceptibility to renal cell carcinoma.4 As mentioned earlier, the VHL alleles associated with Type 2C VHL disease (pheochromocytoma only) encode proteins that retain the ability to polyubiquitinate the HIF-1α subunits.10,11 Presumably, this accounts for the absence of hemangioblastomas and renal cell carcinomas in families with Type 2C VHL disease.41 It would appear from our study that ELS tumors behave in a similar fashion.

Nevertheless, normoxic stabilization of HIF-1 alone, although capable of mimicking some aspects of the VHL loss, is not sufficient to reproduce tumorigenesis, indicating that it is not the critical oncogenic substrate of VHL.57 This is further supported by observations that forced activation of HIF target genes in vivo by HIF-1α variants that escape VHL control does not lead to cyst or tumor formation.15,17 It is important to note, however, that the cells transfected with the overexpression constructs were skin and muscle and therefore possibly not relevant to VHL disease.41

Conclusions

Both the sporadic and VHL-associated ELS tumors in this study had normal VHL-mediated HIF-1 regulation. This was a result of normal VHL gene expression in the patient with the sporadic ELS tumor. In the VHL-associated ELS tumor, this was due to one remaining functional copy of the VHL gene and adequate VHL gene expression. Given the limited number of tumors that have been analyzed both in this study and in previous ones, further work is warranted to determine the role of VHL disease and HIF-1 in tumorgenesis and growth of ELS lesions.

References

Role of VHL genetics, pVHL, and HIF-1α expression in ELS tumors


70. Semenza GL: Physiology meets biophysics: visualizing the interactions of hypoxia-inducible factor 1α with p300 and CBP. Proc Natl Acad Sci USA 99:11570–11572, 2002


Address reprint requests to: Randy L. Jensen, M.D., Ph.D., Department of Neurosurgery, University of Utah, 3B–409 SOM, 30 North 1900 East, Salt Lake City, Utah 84132-2303. email: randy.jensen@hsc.utah.edu.