Effects of VHL Deficiency on Endolymphatic Duct and Sac

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Abstract
The von Hippel-Lindau (VHL) disease is caused by VHL germ line mutation. Inactivation of the wild-type copy of the VHL gene leads to up-regulation of hypoxic response and tumor formation within central nervous system (CNS), kidneys, pancreas, adrenal glands, epididymis, broad ligament, and the endolymphatic sac/petrous bone. Endolymphatic sac tumors (ELST) have been proposed to be derived from endolymphatic sac epithelium, but other possible structures of origin have been implicated. To clarify the anatomic and cellular origin of ELSTs, we did a morphologic and molecular pathologic analysis of 16 tumors. In addition, we investigated effects of VHL deficiency on "tumor-free" endolymphatic duct and sac of VHL patients. Several tumors included in the study were <1 cm in size, and their origin could be placed in the intraosseous portion of the endolymphatic duct/sac. Furthermore, by analysis of clinically uninvolved "tumor-free" endolymphatic duct and sac tissues of VHL patients, we discovered a variety of VHL-deficient microscopic abnormalities with morphologic similarities to ELSTs. We conclude that most, if not all, ELSTs arise within the intraosseous portion of the endolymphatic duct/sac, the vestibular aqueduct. In analogy to renal parenchyma and selected topographical sites within the CNS, endolymphatic duct/sac epithelia are preferentially and multifocally targeted in VHL disease. The primary effect of VHL deficiency on human endolymphatic duct/sac epithelium seems to be the generation of multifocal sites of VHL-deficient cell proliferations from which tumorigenesis may or may not occur. Therefore, inactivation of the VHL wild-type allele seems necessary but not sufficient for the formation of tumor. (Cancer Res 2005; 65(23): 10847-53)

Introduction
Endolymphatic sac tumors (ELST) were identified by Heffner in 1989 (1). They occur sporadically or as a component of autosomal-dominant von Hippel-Lindau (VHL) disease (2–5), a disorder characterized by hemangioblastomas of the central nervous system (CNS) and renal carcinomas as major manifestations. Other manifestations include pheochromocytomas, paragangliomas, pancreatic microcystic adenomas, and epididymal cystadenomas. Inactivation of the VHL tumor suppressor gene also occurs in sporadic ELSTs (6–8) and those associated with VHL disease (9).

The endolymphatic sac and duct are part of the nonsensory membranous labyrinth of the inner ear. Their functional role has not been clarified. Putative functions include maintenance of homeostasis (10, 11) and pressure (12) of the inner ear, phagocytosis of debris (13), and immunologic functions (14, 15). ELSTs can cause hearing loss, tinnitus, vertigo, and facial nerve paresis. These symptoms can arise by infiltration of neighboring structures. It has recently also been suggested that ELSTs can cause symptoms by hemorrhage, endolymphatic hydrops, or both (5, 9).

ELSTs were previously classified as paragangliomas, adenomatous tumors of mixed histology, choristomas, ceruminomas, chordoid plexus papillomas (16), and papillary ependymomas (17). Although Heffner classified them as "low-grade adenocarcinoma of probable endolymphatic sac origin" (1) after analysis of 15 cases, the exact anatomic and cellular origin of these tumors remain unclear. The location of the tumors lead to the conclusion that endothelia of either medial mastoid air cells or the endolymphatic sacs are possible structures of origin (1). Other investigators do not think the endolymphatic sac to be the site of origin. Instead, they suggest the mucosa of the pneumatic spaces surrounding the jugular bulb as site of origin due to the strong positive immunohistochemical reaction for keratin in the tumor tissue (18). Because these tumors express neural-related antigens (S-100, synaptophysin, and Leu7), others suggest that the tumors are of neuroectodermal origin (1, 19). To clarify ELST tumorigenesis, we analyzed a subgroup of small tumors that were large enough to be identified by sensitive imaging studies but small enough to unequivocally reveal their structure of origin.

VHL disease affects a highly selective set of organs, including CNS and kidneys, in which multiple tumors frequently occur. Analysis of preferentially involved sites (e.g., spinal cord and cerebellum) has also revealed evidence of an abundance of microscopic VHL-deficient cellular proliferations that have been proposed to represent precursor material for potential tumor formation (20). In analogy to that approach, we closely examined endolymphatic sac and duct tissue from clinically uninvolved patients to detect potential precursor structures.

Materials and Methods

Tissue
Sixteen ELSTs, resected between 1990 and 2005 at the NIH, were included in the study. Of the 16 tumors, seven were ≤10 mm in largest dimension (range, 3-10 mm; median, 5 mm). Additionally examined were three specimens of tumor-free endolymphatic sac (two specimens obtained at surgery for an ELST confined to the endolymphatic duct and one was from an autopsy). In addition, one specimen of a clinically uninvolved temporal bone was removed during autopsy of a patient with known ELST involving the contralateral endolymphatic sac/duct. All patients had a documented germ line mutation in the VHL gene.
Imaging Studies

Computed tomography imaging. To detect otic capsule invasion and define the extent of bony erosion by ELSTs, temporal bone computed tomography imaging (axial and coronal: 1-1.5 mm slice thickness, 16-22 cm field of view; bone algorithm reconstruction kernel) was done.

Magnetic resonance imaging. Inner ear magnetic resonance imaging was used to detect the tumor in the temporal bone and to detect abnormal signal within the sensory labyrinth, using 1.6-mm overlapping T₂-weighted before and after contrast. T₂-weighted fast induction of steady-state acquisition, and 3 to 5 mm fluid attenuated inversion recovery. Image resolution was <1 mm in plane in all cases.

Microscopic Evaluation and Immunohistochemistry

The following protocol was used for detection of expression of neuron-specific enolase (NSE), cytokeratin AE1/3, cytokeratin MAK66, epithelial membrane antigen (EMA), and CD34. Serial sections were taken from paraffin-embedded tissue blocks for histology and immunohistochemistry. Sections from petrous bone were obtained after prolonged decalcification of formalin-fixed material according to protocols established by the Massachusetts Eye and Ear Infirmary. For antigen retrieval, sections were treated with DAKO Target Retrieval Solution (DAKO, Carpinteria, CA) and incubated at 95°C for 20 minutes. Sections were cooled at room temperature and washed thrice in PBS. After three washes in PBS, sections were incubated in 10% horse serum for 1 hour. The primary antibody was diluted in 2% horse serum, and the sections were incubated in a humidified chamber at 4°C overnight. Primary antibodies used included anti-human NSE, AE1/3, EMA, CD34 (DAKO), S100 (Biogenex, San Ramon, CA), and MAK66 (Zymed, South San Francisco, CA). The sections were then incubated with secondary antibody and avidin-biotin complex for 1 hour each. The reaction product was visualized with 3,3'-diaminobenzidine. A 30-second counterstaining with Mayer's hematoxylin followed. Sections were dehydrated by graded ethanol washes of 95% and 100% and washed with xylene before being mounted.

A modified protocol was used for detection of expression of hypoxia-inducible factor-1 (HIF-1), HIF-2, and carbonic anhydrase IX (CAIX). For antigen retrieval, sections were immersed in preheated DAKO target retrieval solution in a pressure cooker for 90 seconds. Antigen retrieval was not necessary for CAIX labeling. Primary antibodies were mouse monoclonal anti-human HIF-1α human HIF-1α (Neomarkers, Fremont, CA), 1:1,000; rabbit polyclonal anti-mouse HIF-2α PM8 antiserum (21), 1:10,000; mouse monoclonal anti-human CAIX M75 (22), 1:50; and rabbit anti-human GLUT-1 (DAKO), 1:200. Primary antibody was omitted for negative controls. Antigen/antibody complexes were revealed by means of the Catalyzed Signal Amplification system (DAKO; HIF) or Envision system (DAKO; CAIX and GLUT-1) according to the manufacturer's instructions. Sections were counterstained with hematoxylin for 15 seconds, dehydrated in graded ethanol washes, and mounted in DPX (Lamb, Eastbourne, United Kingdom).

Microdissection and Loss of Heterozygosity Analysis

Five-micrometer tissue sections were serially taken from paraffin-embedded tissue blocks. Structures of interest were visualized after H&E staining and subsequently microdissected from a consecutive slide. Other consecutive slides were used for immunohistochemical analysis. Microdissection was done under direct light microscopic visualization using a 30-gauge needle, as previously described (23). Whereas previous microdissection studies exclusively analyzed tumor material (8), we also microdissected morphologically ambiguous cyst epithelium. Both microdissected tumor cells and cyst epithelium were subjected to PCR-based loss of heterozygosity (LOH) analysis of the VHL locus. Fibrous tissue or dura were obtained by microdissection from the same slide to serve as negative controls. Procured cells were immediately resuspended in 10 to 20 μL of buffer [Tris/HC1 (pH 8), 10 mmol/L EDTA (pH 8.0), 1% Tween 20, and 0.1 mg/mL proteinase K] and incubated at 37°C for 3 days. The mixture was boiled for 10 minutes to inactivate proteinase K, and 1.5 μL of this solution were used for PCR amplification of DNA.

Tumors and cystic lesions identified and obtained from clinically normal endolymphatic sac were analyzed for LOH with the microsatellite marker D3S1038 (Research Genetics, Huntsville, AL), flanking the VHL gene on chromosome 3p25. PCR was done for 35 cycles: denaturing at 95°C for 1 minute, annealing at 55.5°C for 40 seconds, and extending at 72°C for 1 minute. For all primers, the final extension was continued for 10 minutes. Each PCR sample contained 1.5 μL of template DNA, as noted above; 10 pmol of each primer; 20 mmol each of dATP, dCTP, dGTP, and dTTP; 15 mmol/L MgCl₂, 0.1 unit of Tag DNA polymerase; 0.05 μL of [32P]dCTP (6,000 Ci/mmol); and 1 μL of 10×: buffer in a total volume of 10 μL. Labelled amplified DNA was mixed with an equal volume of formamide loading dye (95% formamide, 20 mmol/L EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) and analyzed on a polyacrylamide gel. The samples were denatured for 5 minutes at 95°C and loaded onto a gel consisting of 6% acrylamide (49:1 acrylamide/bis), 5% glycerol, and 0.6× Tris-borate EDTA. Samples were electrophoresed at 60 W at room temperature over 2.5 hours. Gels were transferred to 3-mm Whatman paper and dried, and autoradiography was done with Kodak X-OMAT film (Eastman Kodak, Rochester, NY).

Results

Endolymphatic sac tumors. Imaging of seven small ELSTs (<10 mm) was reviewed. The location of all seven tumors was limited to the intraosseous portion of the endolymphatic sac and endolymphatic duct (vestibular aqueduct; Fig. 1A-B). The tumors were enhanced after i.v. gadolinium administration. Diagnostic features included areas of bone erosion adjacent to the endolymphatic duct and sac. Tumor formation was frequently associated with evidence of resorbed hemorrhage.

Sixteen ELSTs were histologically examined. Tumor sizes ranged from 3 to 30 mm in greatest dimension; seven tumors were 3 to 10 mm in size (median, 5 mm). Regardless of tumor size, three main types of architecture were observed as previously described (1): papillary, cystic, and epitheloid clear cell patterns. Extensively vascularized papillary structures (Fig. 1C) were observed in all 16 tumors. The papillary proliferations were lined by a single row of cuboidal epithelial cells. Prominent pleomorphism was not present, and mitotic figures were rare. Areas of cystic growth (Fig. 1D) were observed in 8 of 16 tumors. The cysts had a single epithelial lining and frequently contained proteinaceous material. One tumor had areas of epithelial clear cell clusters, reminiscent of renal clear cell carcinoma (Fig. 1E). Extensive hemosiderin deposits occurred in half of the tumors (Fig. 1F), and the same tumors had associated degenerative features consisting of fibrosis, inflammation, and cholesterol cleft formation. A feature of all tumors was extensive vascularization (Fig. 1G). By immunohistochemistry, neoplastic epithelial cells were positive for NSE in seven of seven cases (Fig. 1H), for MAK66 in seven of seven cases (Fig. 1I), for AE1/AE3 in seven of seven cases (Fig. 1J), for S100 in six cases, and S100 in one of two cases.

To confirm VHL deficiency in the tumor cells, the expression of HIF-1 and HIF-2, which are known to be immediately up-regulated after loss of VHL function, were investigated by immunohistochemistry. Neoplastic cells abundantly expressed both HIF-1 and HIF-2 (Fig. 2). For further evidence of VHL deficiency, tumor cells were selectively microdissected, and the extracted DNA was subjected to PCR-based LOH analysis (Fig. 2). Informative cases revealed a loss of the wild-type copy of the VHL gene. In addition, we investigated the tumors for the expression of the HIF downstream targets CAIX and GLUT-1. Our results revealed a consistent up-regulation of both CAIX and GLUT-1 in the tumor cells (Fig. 2).

Endolymphatic sac and duct precursor structures. For identification of potential ELST precursor structures, four grossly tumor-free specimens (two surgical and two autopsy specimens) of
endolymphatic duct and sac were analyzed. The specimens of tumor-free endolymphatic sac revealed a variety of irregular microscopic morphologic features resembling those of ELSTs. Irregular morphologic features detected in tumor-free endolymphatic sac included (a) multilocular focal microscopic papillary proliferations characterized by an increase of cuboidal epithelial cells supported by connective tissue that was highly vascular (Fig. 3). Moreover, (b) several small cystic structures with mild irregular epithelium, including variable intensity of nuclear staining and variable intensity of cytoplasmatic staining, were observed in two endolymphatic sac specimens. In one case, a microscopic cluster of (c) clear cell change was present (Fig. 3).

Findings in clinically uninvolved, tumor-free endolymphatic ducts were investigated in a specimen of temporal bone after prolonged decalcification. Examination of close step sections from the block revealed multifocal papillary hyperplasia of the epithelium throughout the entire course of the endolymphatic duct beginning at its connection to the utriculus, extending through the mid portion of the endolymphatic duct, and continuing along the endolymphatic sac (Fig. 4).

Cyst formation may be part of the normal morphologic spectrum of the extraosseous portion of the endolymphatic sac (24). We therefore examined multiple atypical-appearing cystic structures for evidence of VHL inactivation. These irregular cystic structures revealed positive nuclear signal for HIF-1 and HIF-2 of variable intensity, whereas the normal epithelial structures of the endolymphatic sac were negative. For further proof of VHL deficiency, we microdissected these lesions and investigated them for VHL gene inactivation. The lesions revealed loss of the wild-type VHL allele, whereas normal control tissue did not reveal LOH (Fig. 2). In addition, we investigated early lesions for the expression of HIF target proteins CAIX and GLUT-1. Our results revealed a consistent up-regulation of both CAIX and GLUT-1 in the epithelium of the microscopic precursor structures (Fig. 2).

Discussion

Endolymphatic sac tumors. In his original study, Heffner analyzed 20 ELSTs, all of which were large, documented either by direct measurement or by space-occupying effects such as "4th ventricle displacement, massive expansion, brain stem shift, pineal displacement, and extensive bone destruction" (1). Although most tumors investigated in our study were significantly smaller than those described by Heffner, they contained the immunohistologic profile and the spectrum of morphologic heterogeneity that was originally observed (1).

Selected tumors showed nuclear immunoreactivity with anti-HIF-1 and anti-HIF-2, which was exclusively confined to the neoplastic cells and negative in the fibrovascular connective tissue. To obtain further evidence of VHL deficiency in the tumor cells, we microdissected the HIF-positive epithelial structures from a consecutive section from the same paraffin block. The microdissected tumor cells had LOH at the VHL locus, whereas the analysis was negative in adjacent normal dura dissected from the same slide (Fig. 2). We therefore confirmed that (a) ELSTs in
VHL disease have lost the wild-type copy of the VHL gene, and (b) the epithelial cells are the neoplastic component of the tumor (4, 8).

Endolympathic sac and duct precursor structures. Analogous to a recent study on the effects of VHL deficiency on the human CNS (8), we intentionally studied clinically uninvolved, tumor-free endolympathic sac and duct of VHL patients. Although the number of specimens was limited (n = 4), we detected a significant number and variety of lesions that have previously been unappreciated. Endolympathic sac and endolympathic duct specimens revealed multifocal microscopic epithelial abnormalities, including (a) focal papillary proliferations, (b) small cystic structures with irregular epithelium, (c) occasional foci of clear cell change, and (d) multifocal papillary proliferation along the entire intraosseous course of the endolympathic sac and duct.

In interesting analogy to ELST, nontumorous epithelium of microscopic precursor structures revealed evidence of VHL gene inactivation. First, HIF-1 and HIF-2 and both target genes CAIX and GLUT-1 are consistently up-regulated in the epithelium of the microscopic precursor structures. Second, we have directly shown inactivation of the wild-type copy of the VHL gene by PCR-based LOH analysis of microdissected precursor cells.

It is not entirely clear whether the papillary or cystic microscopic lesions represent independent morphologic manifestations of VHL disease in the endolympathic duct and sac epithelium. The morphologic spectrum of these microscopic lesions, however, closely resembles the morphologic spectrum observed within ELSTs, which suggests that they represent potential precursor material for ELSTs. Therefore, analogous to the effects of VHL deficiency in human nerve roots, we suggest widespread and multifocal selective involvement of endolympathic sac and duct epithelium with microscopic cellular proliferation to be an effect of VHL germ line mutation, associated with multiple second hits. Inactivation of the VHL wild-type allele seems necessary but not sufficient for the formation of tumor in VHL disease. It is of interest that a specific VHL gene mutation (VHL 598C>T) has been identified causing elevated levels of vascular endothelial growth factor, a VHL target protein, in serum of patients with homozygous mutations (25). The phenotype of VHL598C>T, Chuvash polycythemia, is not associated with formation of any VHL disease-associated tumor and therefore markedly distinct from classic VHL disease. A significant increase of vertebral hemangiomas has been documented in patients with Chuvash polycythemia. The origin of these hemangiomas remains unexplained but is expected to be pathogenetically distinct from that of hemangioblastomas, which occur in classic VHL disease. This and other studies have shown VHL disease to be characterized by multifocal deposits of developmentally arrested precursor material in target organs (20). Unless microscopic precursor sites could be shown in tissues of patients with Chuvash

Figure 2. Evidence for VHL deficiency in ELST and tumor-free VHL endolympathic sac. Neoplastic epithelium of ELSTs (A) consistently revealed positive immunoreactivity with anti-HIF 1 and anti-HIF 2 (B-C). Immunohistochemistry for HIF target proteins CAIX and GLUT-1 was consistently positive in ELSTs (D-E). Irregular-appearing cystic structures in a tumor-free endolympathic sac specimen of a VHL patient (F) stain positive for HIF-1 and HIF-2 (G-H), as well as for target proteins CAIX and GLUT-1 (I-J). Epithelial cells of tumor and cystic structures in tumor-free endolympathic sac were selectively microdissected (K) for LOH analysis (L). Adjacent dura was microdissected for negative control. For deletion analysis, DNA from microdissected tissue samples of the lesions and respective normal controls was amplified by PCR using primers for the polymorphic marker D3S1038 at 3p25 in close vicinity of the VHL gene, and analogic analogous VHL gene mutation (VHL 598C>T) has been identified causing elevated levels of vascular endothelial growth factor, a VHL target protein, in serum of patients with homozygous mutations (25). The phenotype of VHL598C>T, Chuvash polycythemia, is not associated with formation of any VHL disease-associated tumor and therefore markedly distinct from classic VHL disease. A significant increase of vertebral hemangiomas has been documented in patients with Chuvash polycythemia. The origin of these hemangiomas remains unexplained but is expected to be pathogenetically distinct from that of hemangioblastomas, which occur in classic VHL disease. This and other studies have shown VHL disease to be characterized by multifocal deposits of developmentally arrested precursor material in target organs (20). Unless microscopic precursor sites could be shown in tissues of patients with Chuvash
polycythemia, it is likely that the absence of tumorigenesis in Chuvash polycythemia is primarily due to absence of precursor material described in the VHL syndrome.

Anatomic origin of endolymphatic sac tumors. Classification of ELSTs previously included a large variety of tumor types arising from middle ear structures, including tympanic epithelium, Eustachian epithelium, paraganglia, etc. (26, 27). Heffner separated temporal bone tumors of probable endolymphatic sac origin from tumors that arise from a variety of anatomic structures of the middle ear (1). However, it has not been clear whether all “ELSTs” are derived from the inner ear, or if neuroectodermal middle ear tissue can give rise to indistinguishable neoplasms (26).

Traditionally, the endolymphatic duct/sac system has been described as a single-lumen tubular structure with a long thin endolymphatic duct ending in a short, blunt, and pouch-like endolymphatic sac (28, 29). The exact border between endolymphatic duct and sac is not clearly defined and is the subject of discussion. Recently, the endolymphatic duct has been suggested to be a short single lumen tubule only 2 mm long (24, 30). The endolymphatic sac is variable in size and irregular in outline. In general, the intraosseous part of the endolymphatic duct/sac system is referred to as the vestibular aqueduct (31).

Proximodistally, the endolymphatic duct/sac epithelium covers the endolymphatic duct and the intraosseous and extraosseous portion of the endolymphatic sac (24). All our tumors >10 mm in diameter were associated with bone erosion, presumably due to an intraosseous origin. Furthermore, all tumors resected between 1990 and 2005 of ≤10 mm in largest dimension were located within the intraosseous portion of the endolymphatic duct and sac. Although the exact intraosseous anatomic border between endolymphatic duct and sac may be difficult to define in an individual patient, this study clearly shows that VHL deficiency induces cellular proliferation along the entire course of the endolymphatic duct and sac epithelium, from the utricular ostium to the extraosseous portion of the endolymphatic sac (34). The designation endolymphatic “sac” tumor, therefore, does not include the anatomic origin of several of the tumors in this series, because it does not include the possibility of endolymphatic duct origin. Instead, a more accurate designation would be “endolymphatic duct/sac tumors” to include origin along endolymphatic duct and sac. Alternatively, “papillary cystadenoma of the vestibular aqueduct” would include both endolymphatic duct and sac origin and would reflect the primarily intraosseous origin of these tumors. This nomenclature would also emphasize the importance of targeting therapy of these tumors to include not only the endolymphatic sac but also the endolymphatic duct.

The histopathology of the tumors included in this study was strikingly similar with that of benign cystadenomas of the pancreas and epididymis that frequently occur in VHL (32, 33). Furthermore, malignant tumor formation in the VHL kidney may also be preceded by benign cyst formation. The frequent, if not exclusive, origin of these tumors within the intraosseous portion of the endolymphatic sac/duct explains the tendency of bone erosion that is almost universally associated with ELSTs (34). It is therefore not clear to what extent bone erosion is a function of aggressive biological behavior or is merely a function of intraosseous topography.
The aggressive biological behavior of ELSTs has been suggested to be responsible for an unusually high rate of local recurrence after surgery. However, increased rates of postoperative recurrence could also be explained by incomplete resection due to a lack of awareness of possible endolymphatic duct origin, or by development of a second tumor from retained endolymphatic duct/sac epithelium.

The observation of abnormal epithelium lining in the vestibular aqueduct in patients with normal computed tomography and MRI may also explain the high frequency of vestibular symptoms, which has been reported in VHL patients with no radiologically evident tumor (5).

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