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# Allelic Deletion and Mutation of the von Hippel-Lindau (VHL) Tumor Suppressor Gene in Pancreatic Microcystic Adenomas

Alexander Oliver Vortmeyer,* Irina A. Lubensky,* Franz Fogt,* W. Marston Linehan,† Urmila Khettry,‡ and Zhengping Zhuang*

*From the Laboratory of Pathology* and Urologic Oncology Branch,† National Cancer Institute, Bethesda, Maryland, and Deaconess Hospital,‡ Boston, Massachusetts*

**An association between pancreatic microcystic (serous) adenomas (MCAs) and von Hippel-Lindau (VHL) disease has been suggested. However, genetic alterations of the VHL gene in MCAs of the pancreas have never been reported. In this study, we performed genetic analysis of 12 pancreatic MCAs. In 2 cases, VHL disease was documented clinically, and 10 cases were sporadic. For LOH analysis, tumor and normal pancreatic cells were procured from formalin-fixed, paraffin-embedded material using tissue microdissection. After DNA extraction, the samples were amplified by polymerase chain reaction using the polymorphic markers D3S2452, D3S1110, D3S192, and D3S656. In addition, the sporadic tumors were analyzed for VHL gene mutations using probes 3b/10b and K55/K56. Both MCAs associated with VHL disease showed LOH with at least one of the microsatellite markers tested. Among the 10 sporadic cases, 7 tumors showed LOH at the VHL gene locus. A somatic VHL gene mutation on exon 2 was documented in one sporadic case. The study provides the first direct genetic evidence for the role of the VHL gene in MCA tumorigenesis. Furthermore, VHL gene alterations may be detected in both VHL-associated and sporadic pancreatic MCAs.** *(Am J Pathol 1997, 151:951–956)*

Microcystic adenomas (serous cystadenomas) of the pancreas (MCAs) are benign tumors composed of small cysts lined by flattened or cuboidal, glycogen-rich cells.[1] The histogenetic origin of the cystadenoma cells is unclear. Histological and ultrastructural studies suggested derivation from centroacinar cells[1–3] or ductal epithelium.[3–5] The tumor may be detected incidentally or present with obstructive biliary or pancreatic disease.[2]

An association of cystic pancreatic disease and von Hippel-Lindau (VHL) disease has been suggested.[6,7] In VHL-disease-associated pancreatic cystic disease, the mean patient age at presentation is considerably lower compared with the age of presentation in sporadic disease.[8,9] Histologically, pancreatic disease in VHL patients may present as purely cystic disease or as microcystic adenoma.[1,9] Because most VHL patients with pancreatic cystic disease are managed conservatively, the majority of reports on pancreatic cystic disease in VHL patients are based on ultrasound or computer tomography imaging studies.[9,10] It is, therefore, unknown what percentage of VHL patients with cystic pancreatic disease have, in fact, MCA. Furthermore, it is currently unclear whether pancreatic cysts and microcystic adenomas are different entities or represent the same disease process.[9]

The VHL tumor suppressor gene has recently been mapped to chromosome 3p25[11] and subsequently identified.[12] Genetic changes of the VHL gene, however, have never been studied in MCAs. In this study, we analyzed a series of 12 MCAs for the presence of allelic deletions and somatic mutations at the VHL gene. We used tissue microdissection to selectively procure the cyst-lining cells and minimize contamination by somatic cells. Both VHL-disease-associated and sporadic cases were analyzed in this study.

## Materials and Methods

### Patients and Tumors

Twelve cases of MCA consisting of 11 surgical and 1 autopsy specimen were retrieved from the files of the Laboratory of Pathology, National Cancer Institute, National Institutes of Health, and the New England Deaconess Hospital, Harvard Medical School. Two specimens, one autopsy (case 1) and one surgical pathology specimen (case 2), were from patients with VHL disease (Table 1). Patient 1 died at the age of 40 from VHL-disease-related causes, and the pancreatic MCA was incidentally found at autopsy. Germline mutation analysis had never been performed in this patient. Patient 2 was 38 years old when a pancreatic MCA was detected. Subsequent germline mutation analysis revealed a C to T transversion at nucleotide 694 leading to frameshift mutation (arg to stop). Both patients with VHL disease had other VHL-

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Address reprint requests to Dr. Irina A. Lubensky, Laboratory of Pathology, National Cancer Institute, NIH, Building 10, Rm 2N212, 9000 Rockville Pike, Bethesda, MD 20892.

**Table 1.** Patients with Microcystic Adenomas: Clinical Data

| Case | Age (years) Sex | VHL disease-associated tumors |
|---|---|---|
| 1 | 40 M | Cerebellar hemangioblastoma, renal cell carcinoma, pheochromocytoma |
| 2 | 38 M | Renal cell carcinoma, pheochromocytoma |
| 3 | 69 F | None |
| 4 | 62 M | None |
| 5 | 51 F | Not known |
| 6 | 83 F | None |
| 7 | 50 F | Not known |
| 8 | 66 F | Not known |
| 9 | 64 F | Not known |
| 10 | 65 F | None |
| 11 | 64 F | None |
| 12 | 66 F | None |

M, male; F, female.

disease-associated tumors (Table 1). Ten specimens (cases 3 to 12) were from patients (one male and nine females; mean/median age, 64/65 years) who had no evidence and no family history of VHL-disease-associated neoplasms. All tumors showed the characteristic gross and microscopic features of MCA (Figure 1).

## *Microdissection*

Unstained 5-$\mu$m, formalin-fixed, paraffin-embedded tissue sections on glass slides were deparaffinized twice with xylene, rinsed with graduated ethanol from 100 to 70%, briefly stained with hematoxylin and eosin (H&E), and rinsed in 10% glycerol in Tris-EDTA buffer. A modified microdissection procedure was performed under direct light microscopic visualization using a 30-gauge needle as previously described.[13] Cyst-lining epithelial cells were selectively dissected, and procurement of subjacent fibrovascular stroma was avoided (Figure 1). Control samples from normal pancreatic tissue were obtained from the same slide in all cases.

## *DNA Extraction*

Procured cells were immediately resuspended in 10 to 20 $\mu$l of buffer containing Tris/HCl, pH 8.0, 10 mmol/L ethylenediamine tetra-acetic acid, pH 8.0, 1% Tween 20, and 0.1 mg/ml proteinase K and were incubated at 37°C overnight. The mixture was boiled for 5 minutes to inactivate the proteinase K and 1.5 $\mu$l of this solution was used for polymerase chain reaction (PCR) amplication of DNA.

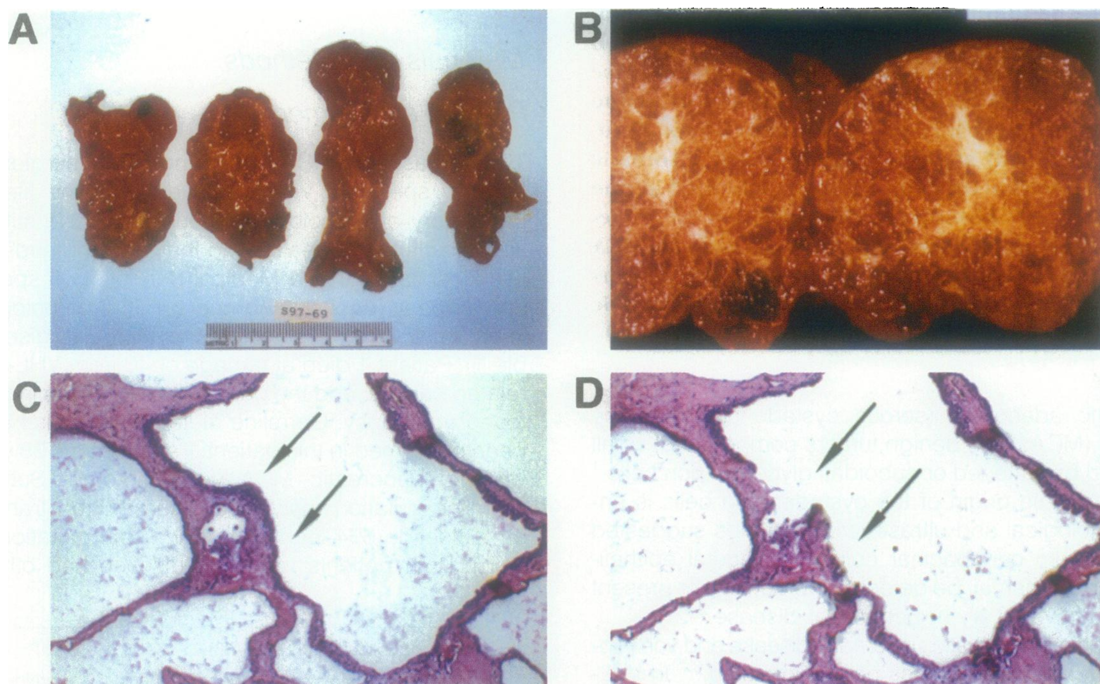


**Figure 1. A:** Gross photograph of MCA in VHL patient. **B:** Gross photograph of MCA in sporadic case. **C** and **D:** MCA (H&E stain; magnification ×10 before (**C**) and after (**D**)) microdissection of tumor cells. **Arrows** indicate area of cell procurement.

**Table 2.** LOH Analysis at the VHL Gene Locus in 12 Pancreatic MCAs with Five Genetic Markers

| Case | D3S2452 (3p14–21) | D3S192 (3p25) | D3S1110 (3p25) | 104/105 (intragenic) | D3S656 (3p25) |
|---|---|---|---|---|---|
| 1 | – | ○ | ● | – | ● |
| 2 | ○ | ○ | ● | ● | – |
| 3 | – | ○ | ○ | ○ | ○ |
| 4 | – | ● | ● | – | ● |
| 5 | – | – | – | – | – |
| 6 | – | – | ○ | ● | ○ |
| 7 | ○ | ● | ○ | – | ○ |
| 8 | – | – | – | – | – |
| 9 | ○ | ● | ● | ● | ○ |
| 10 | ○ | ○ | – | – | ○ |
| 11 | ○ | ○ | ○ | – | ● |
| 12 | ○ | ○ | ● | – | ○ |

–, not amplified or not informative; ○, no LOH; ●, LOH.

## *Loss of Heterozygosity (LOH) Analysis*

Twelve cases (tumor and control tissue) were examined for LOH with four microsatellite markers (D3S2452, D3S1110, D3S192, and D3S656) and one intragenic polymorphic marker upstream of the coding region of the VHL gene (104/105: upstream, 5′AGT GGA AAT ACA GTA ACG AGT TGG CCT 3′, and downstream, 5′GTC CCA GTT CTC CGC CCT CCG GGG CAT 3′.[14] Each PCR sample contained 1.5 μl of template DNA as described above, 10 pmol of each primer, 20 nmol each of dATP, dCTP, DGTP, and DTTP, 15 mmol/L $MgCl_2$, 0.1 U of *Taq* DNA polymerase, 0.05 μl of [$^{32}$P]dCTP (6000 Ci/mmol), and 1 μl of 10X buffer in a total volume of 10 μl. For the four microsatellite markers, PCR was performed with 35 cycles: denaturing at 95°C for 1 minute, annealing at 55°C for 1 minute, and extending at 72°C for 1 minute. The conditions for 104/105 were denaturing at 95°C for 45 seconds, annealing at 61°C for 40 seconds, and extending at 72°C for 40 seconds. For all primers, the final extension was continued for 10 minutes. Labeled amplified DNA (1.5 μl) was mixed with an equal volume of formamide loading dye (95% formamide, 20 mmol/L EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol). The samples were denatured for 5 minutes at 95°C and loaded onto a 6% polyacrylamide gel containing 7 mmol/L urea (D3S2452, D3S1110, D3S192, and D3S656) or a single-strand conformation polymorphism (SSCP) gel (MDE, AT Biochem, Malvern, PA) with 10% glycerol (104/105). After electrophoresis, the gels were transferred to 3-mm Whatman paper and dried, and autoradiography was performed with Kodak X-OMAT film (Eastman Kodak, Rochester, NY). LOH was scored as complete or nearly complete absence of one allele in tumor DNA as defined by direct visualization.

## *Mutation Analysis and DNA Sequencing*

With the 10 sporadic cases 3 to 12 (Table 1), PCR amplification of VHL gene coding sequences with 3b/10b and K55/K56[15] and SSCP analysis were performed with exactly the same conditions as described above (104/105). After detection of aberrant bands in case 5, DNA was extracted from excised bands of the SSCP gel by overnight incubation in 100 μl of distilled water at room temperature and subsequently PCR amplified. The amplified PCR products were used for DNA sequencing (cyclin sequencing kit, Perkin Elmer, Norwalk, CT).

# *Results*

DNA was successfully procured from cyst-lining cells of 12 archival MCAs that were microdissected to avoid or minimize contamination with subjacent stromal tissue (Figure 1). Extracted DNA was PCR amplified with four microsatellite markers (D3S2452, D3S1110, D3S192, and D3S656) and one polymorphic intragenic marker (104/105) upstream of the coding region of the VHL gene.[14] Both VHL-disease-associated MCAs showed 3p25 LOH (Table 2; Figure 2A). Among the 10 sporadic cases, allelic deletions at 3p25.5 were detected in 6 of 10 informative cases at one or more loci (Table 2; Figure 2A). LOH was identified in one additional case (case 5) after SSCP analysis with primers K55/K56 for exon 2 (see below).

VHL gene mutation analysis of the 10 sporadic tumors was performed using SSCP gel electrophoresis with the markers 3b/10b for exon 1 of the VHL gene and K55/K56 for exon 2 of the VHL gene.[15] In one case (case 5), aberrant bands were observed with primers K55/K56 for exon 2 (Table 2; Figure 2, B and C). Sequencing analysis of the bands revealed a T to C transversion of nucleotide 576. This mutation was present only in the cyst cell sample and not seen in the control sample taken from normal pancreatic tissue.

# *Discussion*

VHL disease, an autosomal dominant genetic disorder, is characterized by the development of a variety of neoplasms including hemangioblastomas of the central nervous system, renal cell carcinomas, pheochromocytomas, and pancreatic and renal cysts.[10,16] The VHL tumor suppressor gene has recently been linked to chromosome 3p25[11] and subsequently identified.[12] Recent genetic studies on renal cell carcinomas,[14,17]

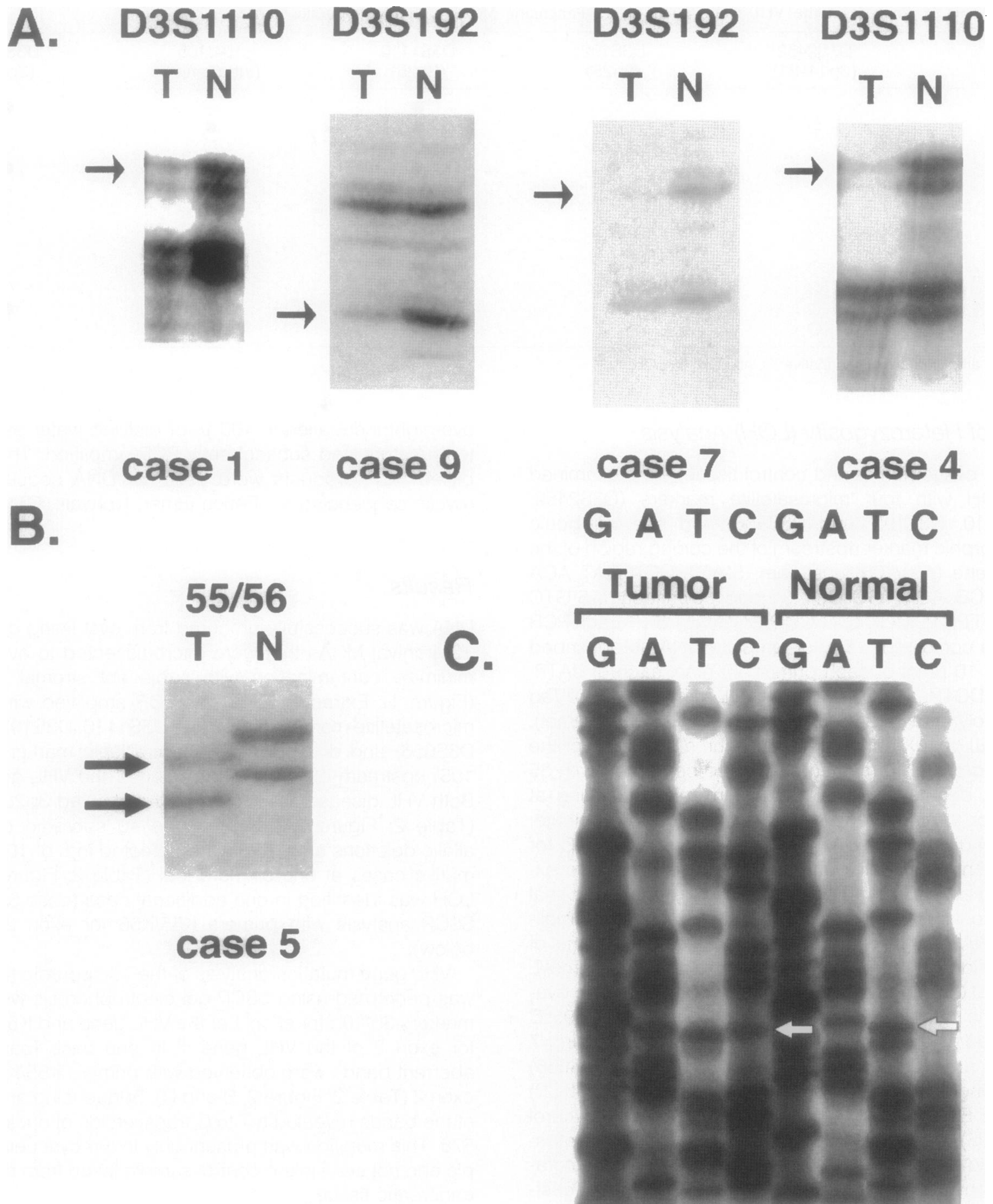


**Figure 2. A:** Representative results of LOH at the VHL gene locus with four genetic markers in four MCAs. All cases show deletion of one allele in tumor tissue (T, **arrows**) as compared with normal tissue (N). **B:** Aberrant bands in tumor tissue of case 5 after amplification with K55/K56 (exon 2) and SSCP analysis. **Arrows** indicate aberrant electrophoresis bands. **C:** Sequencing analysis of tumor and normal bands. **Arrow** indicates T to C transversion at nucleotide 576 in tumor tissue.

pheochromocytomas,[17–19] and hemangioblastomas[20–22] from patients with VHL disease support Knudson's hypothesis[23] that both an inherited germline mutation and loss of function of the wild-type allele of the VHL gene are essential for the development of these neoplasms.

In this study we performed genetic analysis of two microcystic adenomas from known VHL patients. One of the VHL patients had a known germline mutation at exon 3, nucleotide 694 (case 2). In both tumors, microsatellite marker analysis demonstrated LOH at 3p25. We conclude that in both cases the germline mutation of one

allele and deletion of the opposite allele played an important role in the pathogenesis of these tumors.

In sporadic neoplasms, tumorigenesis is thought to be initiated by somatic alteration of both alleles of a tumor suppressor gene.[24] Accordingly, allelic deletions at 3p25 and mutations of the VHL gene have been well documented in sporadic renal cell carcinomas.[14,25,26] Furthermore, somatic mutations have recently been suggested to play a role in a subgroup of sporadic hemangioblastomas.[20–22] Papillary cystadenoma of the epididymis may also occur as a manifestation of VHL disease, and somatic VHL gene mutations were recently detected in a sporadic type of this tumor.[27] Of note, microcystic pancreatic adenoma, renal cell carcinoma, hemangioblastoma, and papillary cystadenoma of the epididymis may exhibit striking morphological similarities to each other.

Six of ten sporadic cases revealed allelic 3p25 deletion with polymorphic microsatellite markers; one additional case (case 5) demonstrated loss of the wild-type allele after amplification with K55/K56 and SSCP gel analysis. Deletion of 3p25 was therefore detected in 7 of 10 (70%) sporadic microcystic adenomas analyzed in this study. Most cases were noninformative for the 104/105 marker, which recognizes a gene segment immediately upstream of the VHL gene. None of the VHL cases and none of the sporadic cases showed LOH with the D3S2452 marker mapped to 3p14–21, which is distant from the VHL 3p25 area. D3S1110, D3S192, and D3S656 have all been mapped to 3p25. Because these three markers are flanking the VHL gene, they may fail to reveal deletions that are confined to the VHL gene or extend only slightly outside the VHL gene area. Thus, the rate of LOH of the VHL gene region may in fact exceed the minimal rate of 70% from our analysis with five polymorphic markers and one nonpolymorphic marker. Therefore, allelic deletion of the VHL gene area appears to play an important role in the development of sporadic MCA.

A somatic mutation was detected in one case that showed abnormal bands after amplification with primers K55/K56 (Figure 2B). Sequencing analysis revealed a T to C transversion at nucleotide 576 (Figure 2C). Similarly to our result, mutations of exon 2 of the VHL gene were observed more frequently than mutations of exon 1 or exon 3 in sporadic renal cell carcinomas.[14] Although SSCP analysis may fail to detect genomic point mutations,[28] it appears from our findings that a mutation event did not take place in the majority of the sporadic MCA cases, which is consistent with previous studies in sporadic hemangioblastomas.[20,21]

In addition, the sporadic microcystic adenoma with exon 2 mutation revealed loss of the opposite allele as compared with the normal tissue. Therefore, a two-hit mechanism, which would parallel the tumorigenesis of familial, VHL-disease-associated tumors, may account for the development of this individual neoplasm. However, the mechanisms leading to MCA tumorigenesis in most of our cases remain unexplained. Different mechanisms of VHL tumor suppressor gene inactivation may account for the functional loss of the second allele. Herman and coworkers[29] reported hypermethylation of a CpG island of the VHL gene as another important mechanism for inactivation of the VHL gene in renal cell carcinoma. Furthermore, other unknown genetic changes may prove to be relevant for the development of sporadic MCA in the future.

We conclude from this study that genetic changes of the VHL gene play an important role in the pathogenesis of both VHL-disease-associated and sporadic MCAs. In VHL patients, loss of the wild-type VHL gene copy in susceptible target cells within the pancreas appears to represent a major step in the initiation and/or progression of these tumors. Furthermore, VHL gene deletion appears to play an important role in the development of sporadic microcystic adenomas.

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