

Allelic Deletions of the *VHL* Gene Detected in Multiple Microscopic Clear Cell Renal Lesions in von Hippel-Lindau Disease Patients

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Patients with von Hippel-Lindau (VHL) disease develop a spectrum of bilateral clear-cell renal lesions including cysts and renal cell carcinomas (RCCs). VHL gene deletions have been previously reported in VHL-associated macroscopic RCC. Although histological analysis suggests that microscopic cystic lesions in the VHL patients may represent precursors of the RCC, there is at present no direct molecular evidence of their relationship. To investigate the relationship between cystic lesions and RCC, 26 microdissected archival renal lesions from two VHL disease patients were studied for loss of heterozygosity at the VHL gene locus using polymerase chain reaction single-strand conformation polymorphism analysis. The renal lesions included 2 benign cysts, 5 atypical cysts, 5 microscopic RCCs in situ, 5 cysts lined by a single layer of cells, in which RCCs in situ were developing, and 2 microscopic and 7 macroscopic RCCs. Except for a single benign cyst, 25 of 26 renal lesions showed nonrandom allelic loss of the VHL gene. In either of the 2 patients, the same VHL allele was deleted in all of the lesions tested, indicating loss of the wild-type allele and retention of the inherited, mutated VHL allele. The results suggest that all clear-cell lesions in the VHL kidney represent neoplasms and that the loss of the VHL gene occurs early in their development. Atypical and benign cysts most likely represent the initial phenotype in malignant transformation to the RCC. (Am J Pathol 1996, 149:2089–2094)

von Hippel-Lindau (VHL) disease is an autosomal dominant disorder characterized by the development of retinal angiomas, central nervous system hemangioblastomas, pancreatic cysts and tumors, pheochromocytomas, and renal cysts and carcinomas.¹ Renal manifestation of the disease occurs in 66% of the VHL patients.² Patients present with a spectrum of bilateral multifocal renal lesions including benign cysts, atypical cysts, and cystic and solid renal cell carcinomas (RCCs).^{3,4} VHL patients present with RCC at a younger age than those with sporadic RCC and are at risk to develop more lesions with increasing patient age.^{1,2,5} The lesions range from microscopic to macroscopic in size and commonly contain a low-grade clear-cell epithelium.^{3,4,6} Histologically, cysts lined by a single layer of cells are considered benign, those with a lining two to three cell layers thick are classified as atypical, and those lesions with more than three cell layers are classified as RCC (Figure 1).^{3,4} Presently, there is no direct molecular evidence demonstrating the relationship between RCC and benign or atypical cysts.

The VHL disease gene has been localized to the chromosome 3p25.5 and recently cloned.^{7–10} Loss of heterozygosity (LOH) on chromosome 3p has been established in macroscopic RCC in VHL and sporadic patients.^{7,11,12} However, microscopic RCC and benign and atypical cysts have not been previously analyzed for the LOH at the *VHL* gene. To investigate at what stage in the development of microscopic renal lesions the genetic alteration of the *VHL* gene occurs, 26 renal lesions from two informative patients were studied for the deletions at the *VHL* gene locus (3p25.5) using a microdissection technique for formalin-fixed, paraffin-embedded tissue¹³ and polymerase chain reaction (PCR)-based single-

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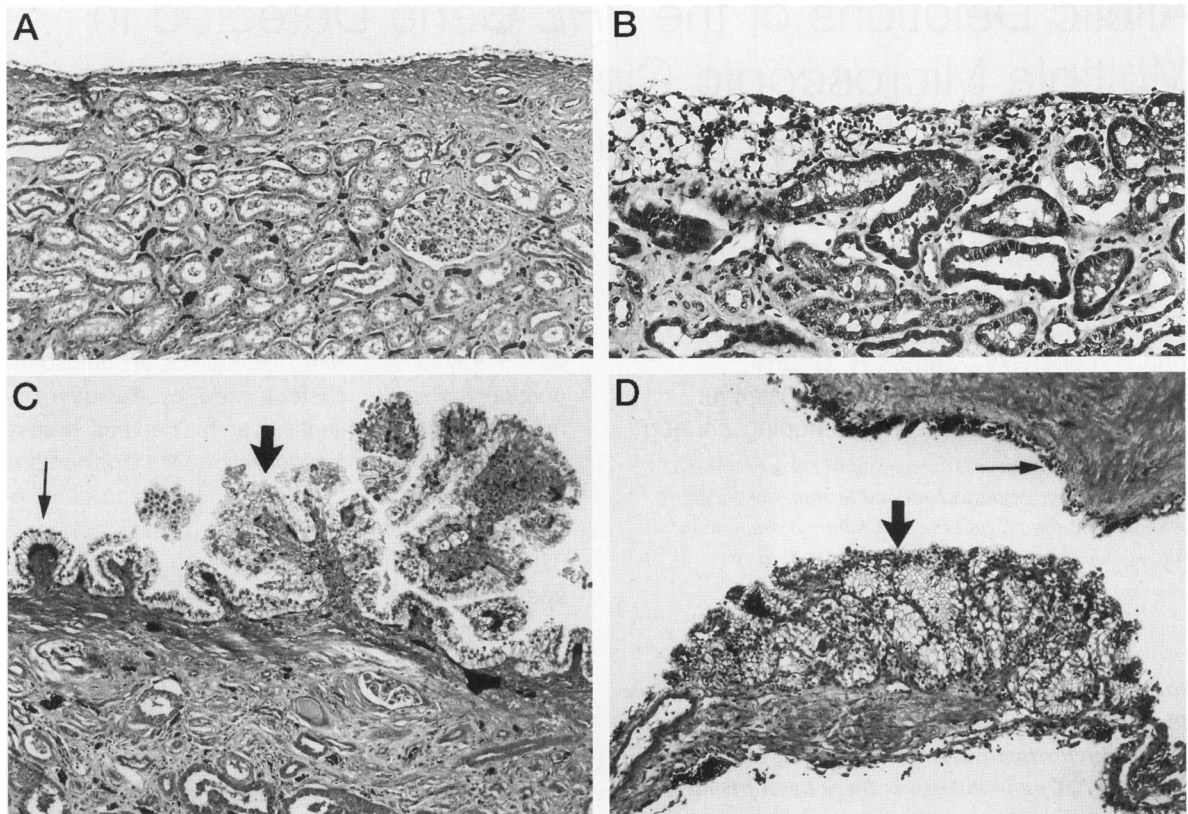


Figure 1. Histology of renal lesions in patient 2 with VHL. **A:** Benign cyst lined by a single layer of clear epithelial cells. H&E stain; magnification, $\times 100$. **B:** Atypical cyst lined by two to three layers of clear epithelial cells. H&E stain; magnification, $\times 100$. **C:** Micropapillary RCCIS developing in a cyst lined by a single layer of cells (arrows). H&E stain; magnification, $\times 100$. **D:** Solid RCCIS (thick arrow) developing in a cyst lined by a single layer of cells (thin arrow). H&E stain; magnification, $\times 100$.

strand conformation polymorphism (SSCP) analysis.¹⁴

Materials and Methods

Patients

Two informative VHL patients in whom normal tissue DNA was heterozygous for a polymorphic marker at the *VHL* gene locus¹⁴ were selected from the group of familial VHL patients with RCC who underwent renal-sparing surgery or nephrectomy at the National Cancer Institute, National Institutes of Health.¹⁵ Patient 1 was a 30-year-old white male (kindred 3624) who was diagnosed with VHL disease at age 11 and underwent bilateral partial nephrectomies in 1992. Patient 2 was a 39-year-old Hispanic male (kindred 3315) who was diagnosed with VHL disease and underwent right radical nephrectomy and left partial nephrectomy in 1992 (Figure 2). Each patient had a documented germ-line mutation for the *VHL* gene.¹⁶

Tissue Microdissection

Formalin-fixed, paraffin-embedded tissue samples from 26 renal lesions and normal renal parenchyma in each patient were obtained from the files of the Laboratory of Pathology, National Cancer Institute, National Institutes of Health. A routine 5- μ m histolog-



Figure 2. Intraoperative photograph of the VHL kidney from patient 2 with 11 solid and cystic lesions.

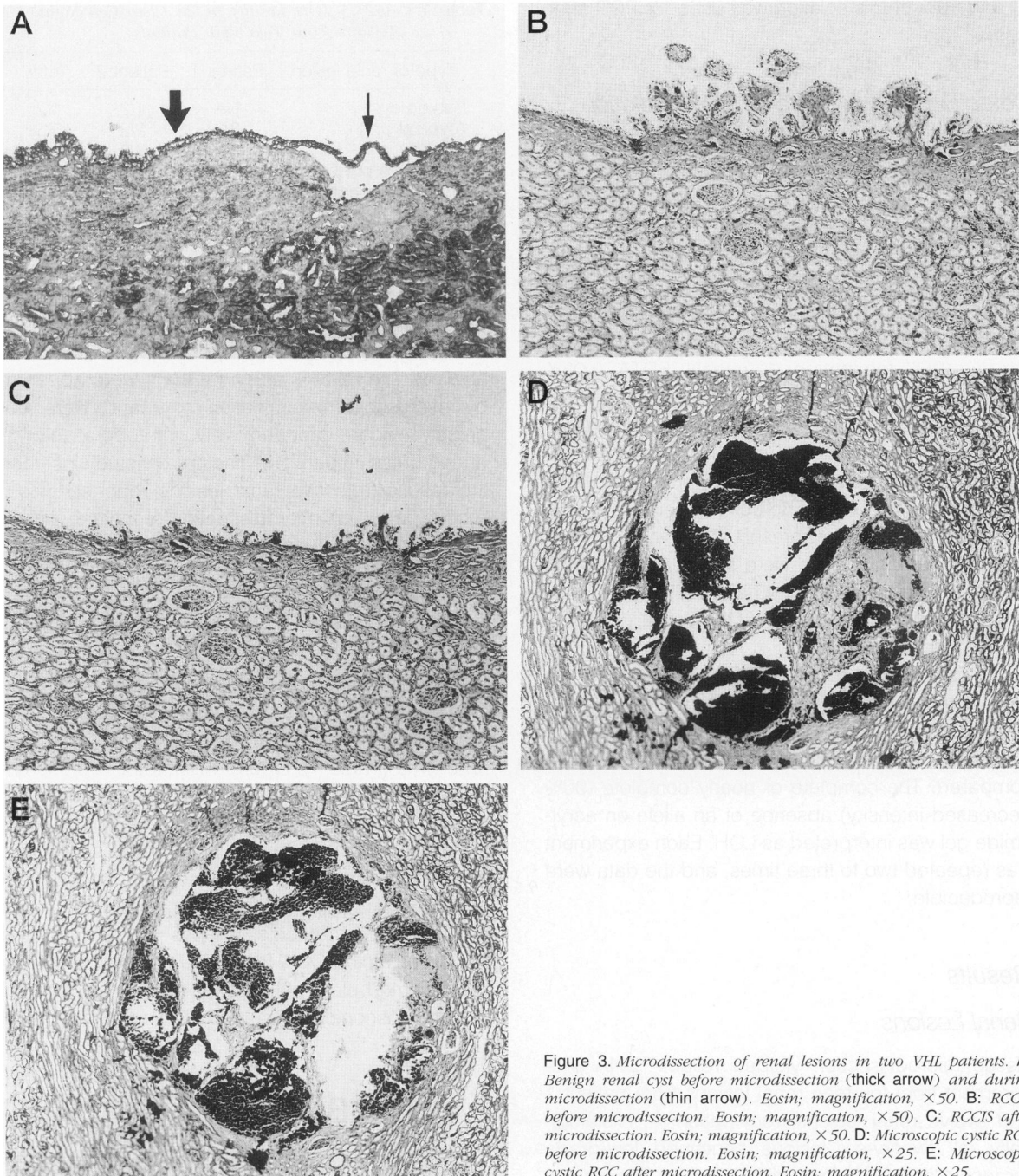


Figure 3. Microdissection of renal lesions in two VHL patients. **A:** Benign renal cyst before microdissection (thick arrow) and during microdissection (thin arrow). Eosin; magnification, $\times 50$. **B:** RCCIS before microdissection. Eosin; magnification, $\times 50$. **C:** RCCIS after microdissection. Eosin; magnification, $\times 50$. **D:** Microscopic cystic RCC before microdissection. Eosin; magnification, $\times 25$. **E:** Microscopic cystic RCC after microdissection. Eosin; magnification, $\times 25$.

ical section was stained with hematoxylin and eosin (H&E) and used for a control. The adjacent serial one to three 5- μm sections were used for microdissection. Normal renal tubular epithelial, cyst-lining, or tumor cells were selected from the 5- μm -thick eosin-stained slides and microdissected using a modified Pasteur pipette under direct light microscopic visualization as described previously (Figure 3).¹³

DNA Extraction

Procured cells were immediately resuspended in a 10- μl solution containing Tris/HCl, pH 8.0, 0.1 mol/L ethylenediamine tetra-acetic acid (EDTA), pH 8.0, 1% Tween 20, and 0.1 mg/ml proteinase K and incubated for 2 hours or overnight at 37°C. The mixture was boiled for 10 minutes to inactivate the proteinase

K, and 10% of this solution was used for PCR analysis.

Detection of VHL Gene (3p25.5) Deletion

The PCR amplification reaction was carried out for 30 cycles at 95°C for 30 seconds and 70°C for 30 seconds, using primers to the polymorphism upstream of the coding region of the *VHL* gene: 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 Labeled amplified DNA was mixed with an equal volume of formamide-loading dye (95% formamide, 20 mmol/L EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol) and analyzed on SSCP gel.¹⁴ 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 0.6X Tris-buffered ethanolamine. Samples were electrophoresed at 8W at room temperature overnight. Gels were transferred to 3-mm Whatman paper and dried, and autoradiography was performed with Kodak X-OMAT film (Eastman Kodak, Rochester, NY).

The case was considered to be informative for the *VHL* gene polymorphism when normal tissue DNA showed two different alleles (heterozygosity). The intensities of the two alleles in the renal lesion were compared. The complete or nearly complete (90% decreased intensity) absence of an allele on acrylamide gel was interpreted as LOH. Each experiment was repeated two to three times, and the data were reproducible.

Results

Renal Lesions

Tissue microdissection yielded reliable DNA procurement from fourteen renal lesions from patient 1 and twelve renal lesions from patient 2. Histologically, all twenty-six lesions contained low-grade (grades 1 and 2)¹⁷ clear epithelial cells. The lesions were classified according to the current classification of the *VHL* tumors³ (Table 1). Two cysts with one-cell-thick linings were classified as benign, five cysts with linings two to three cells thick were considered atypical, and nine lesions lined with more than three cell layers were classified as RCCs (Figure 1 and 3). Five microscopic papillary and solid renal lesions developing in five cysts lined by a single layer of cells were classified as RCC *in situ* (RCCIS; Figure 1). The microscopic RCCIS and corresponding one-cell-thick clear epithelial linings of

Table 1. *3p25.5 LOH Results in 26 Clear-Cell Renal Lesions from Two VHL Patients*

Type of renal lesion	Patient 1	Patient 2	Total
Benign cyst	NA	1/2	1/2
Atypical cyst	3/3	2/2	5/5
RCCIS	2/2	3/3	5/5
Cyst lining of RCCIS	2/2	3/3	5/5
RCC (microscopic)	2/2	NA	2/2
RCC (macroscopic)	5/5	2/2	7/7
Total	14/14	11/12	25/26

NA, not available.

the cysts were analyzed separately (Table 1). Two atypical, five RCCIS, and two RCCs were apparent by microscopic examination only and were not grossly evident preoperatively, intraoperatively, or during gross inspection.⁶ The current study includes the results from two renal lesions reported in our recent paper on microdissection technique developed specifically for the analysis of the microscopic cystic lesions.¹³

LOH Results

In each of the two patients, *VHL* gene LOH was found in DNA from 25 of 26 renal lesions and not in the DNA extracted from adjacent normal renal parenchyma (Table 1). Figure 4 illustrates representative results of SSCP analysis of DNA amplified across the polymorphic site at the *VHL* gene from 4 renal lesions in patient 2. Both alleles are present in the lane N containing DNA procured from the adjacent normal kidney parenchyma. In contrast, loss of the upper allele is detected in lanes BC, AC, IS, and RCC, containing DNA from pure populations of microdissected clear cells from benign cyst, atypical cyst, microscopic RCCIS, and RCC, respectively. All

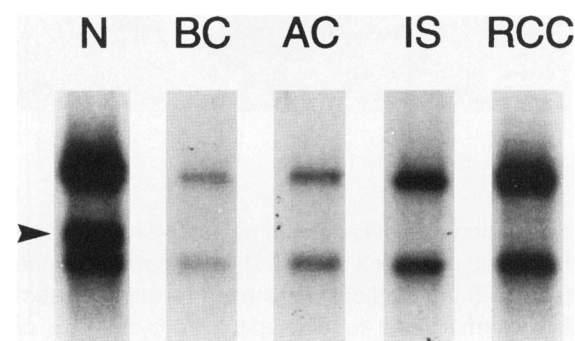


Figure 4. Representative results of the SSCP analysis in four clear-cell renal lesions of the *VHL* patient 2. Loss of the upper allele (arrow) was detected at the *VHL* gene locus on chromosome 3p25.5 in benign cyst (BC), atypical cyst (AC), RCCIS (IS), and RCC as compared with normal kidney (N).

lesions in each patient showed deletions of the same allele.

Discussion

The VHL disease tumor suppressor gene has been localized to the chromosome 3p25.5 and recently cloned. The two-hit theory of Knudson¹⁸ predicts that, in a familial cancer syndrome such as VHL, the genotype of each neoplasm should consist of an allele with an inherited germ-line mutation and loss of function of the inherited wild-type allele through chromosomal deletion or point mutation. Therefore, LOH or mutation at the *VHL* gene should be detectable in the renal lesion if the lesion represents a neoplasm. LOH at chromosome 3p has been reported in VHL-associated and sporadic RCC.^{7,11,12}

Histological evaluation of many radiologically and grossly benign cysts from VHL patients revealed neoplastic foci in the cyst wall.^{2,6} Moreover, the consecutive 5- μ m histological sections of a cyst lined by a single layer of cells may occasionally reveal a developing RCCIS in the wall (unpublished observation). Microscopic foci of RCCs and atypical cysts were found in grossly normal kidney parenchyma adjacent to macroscopic RCC lesions.⁶ In an average VHL kidney, the estimated number of clear-cell benign and atypical cysts and RCCs may be as high as 1100 and 600, respectively.⁶ Immunohistochemistry analysis of clear-cell epithelium in RCCs and atypical cysts in VHL patients reported by Kragel et al demonstrated that both types of lesions may arise from the epithelium of the proximal tubule.¹⁹

Although clinical, radiological, and pathological data suggested that benign and atypical renal lesions may represent the precursors of RCC in patients with VHL disease,^{2-4,6,15,20,21} the genetic evaluation of such lesions has not been performed previously. VHL renal lesions are often small and can be visualized and diagnosed only under light microscopy in histological formalin-fixed, paraffin-embedded tissue sections. Furthermore, many cystic lesions in VHL kidney are lined by a single layer of clear epithelial cells. The procurement of pure epithelium from such lesions without contamination with normal somatic and inflammatory cells has been difficult until the development of a modified microdissection method that allows for procurement of selected pure cell populations from lesions less than 1 mm in size including those with a single layer of cells lining the cyst.¹³

In this study, we successfully employed tissue microdissection and PCR-based SSCP analysis and

found identical patterns of allelic loss of the *VHL* gene in the spectrum of multiple microscopic and macroscopic renal lesions in two VHL patients with known germ-line mutations. Of 26 clear-cell renal lesions ranging from cysts lined by a single layer of cells to microscopic RCCIS and RCC to macroscopic RCC, 25 showed loss of the wild-type allele and retention of the inherited, mutated VHL allele. LOH was not detected in one cyst lined by a one-cell-thick epithelium. Technical difficulty in microdissection of a one-cell-thick epithelial layer could contribute to contamination from normal inflammatory and somatic cells adjacent to the cyst.¹³ Alternatively, LOH at the *VHL* gene may not be detectable in all benign cysts. The results provide the first molecular evidence that LOH of the normal VHL disease gene occurs in benign and atypical clear-cell cysts and, therefore, may represent an early event in the development and progression of RCC in VHL patients. The neoplastic nature of microscopic renal lesions should be taken into account in follow-up of VHL patients with renal involvement. Parenchymal-sparing surgery with enucleation of multifocal VHL renal lesions may be justified on the basis of the indolent nature of the disease, slow growth of the renal lesions, and rare metastatic potential of tumors of small size.^{2,15,20,21}

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