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Mosaicism in von Hippel–Lindau Disease: Lessons from Kindreds with Germline Mutations Identified in Offspring with Mosaic Parents

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Summary

von Hippel-Lindau disease (VHL [MIM 193300]) is a heritable autosomal dominant multiple-neoplastic disorder with high penetrance. It is characterized by brain and spinal-cord hemangioblastomas, retinal angiomas, clear-cell renal carcinoma, neuroendocrine tumors and cysts of the pancreas, pheochromocytomas, endolymphatic-sac tumors, and papillary cystadenomas of the epididymis and broad ligament. Although most index cases have a positive family history of VHL, some do not and may represent de novo cases. Cases without a family history of VHL may or may not have a germline mutation in their *VHL* tumor-suppressor gene. We present two cases of VHL mosaicism. In each of two families, standard testing methods (Southern blot analysis and direct sequencing) identified the germline mutation in the *VHL* gene of the offspring, but not in their clinically affected parent. Additional methods of analysis of the affected parents' blood detected the *VHL*-gene mutation in a portion of their peripheral blood lymphocytes. In one case, detection of the deleted allele was by FISH, and, in the second case, the 3-bp deletion was detected by conformational sensitive gel electrophoresis and DNA sequencing of cloned genomic DNA. Mosaicism in VHL is important to search for and recognize when an individual without a family history of VHL has VHL. Patients diagnosed without family histories of the disease have been reported in as many as 23% of kindreds with VHL. Identification of individuals potentially mosaic for VHL will affect counseling of families, and these individuals should themselves be included in clinical screening programs for occult disease.

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Introduction

von Hippel-Lindau disease (VHL [MIM 193300]) is a rare, heritable, autosomal dominant disorder characterized by a predisposition to develop benign and malignant tumors in multiple organ systems (Glenn et al. 1990; Choyke et al. 1995). Individuals at risk may develop tumors of the CNS (hemangioblastomas), eye (retinal angiomas), kidneys (clear-cell renal-cell carcinoma [RCC]), adrenal glands (pheochromocytoma), pancreas (cysts and neuroendocrine tumors), inner ear (endolymphatic sac tumors) (Manski et al. 1997), and broad ligament and epididymis (papillary cystadenomas) (Choyke et al. 1997). The incidence of VHL ranges from 1 in 36,000 to 1 in 45,500 (Maher et al. 1991; Neumann and Wiestler 1991; Maddock et al. 1996) live births in white populations. Although there is nearly complete penetrance by age 65 years (Maher et al. 1991), there are exceptions. Estimated mutation rates range from 1.4×10^{-6} to 4.4×10^{-6} per gene per generation (Maddock et al. 1996). The proportion of cases caused by new mutations is unknown.

Phenotypes vary among families (Glenn et al. 1991; Neumann and Bender 1998), and a VHL classification system based on observed phenotype differences has been proposed (Chen et al. 1995; Choyke et al. 1995). This schema divides VHL into type I (without pheochromocytoma) and type II (with pheochromocytoma), with subcategories IIA (VHL without predisposition to clear-cell RCC) and IIB (VHL with predisposition to clear-cell RCC). These differences in phenotype reflect differences in genotype (Zbar et al. 1996; Stolle et al. 1998).

In 1988 the *VHL* gene was mapped to chromosome 3 (Seizinger et al. 1988) and was isolated by positional cloning in 1993 (Latif et al. 1993). The detection of germline mutations in families has recently been reported to be 100% (Stolle et al. 1998) on the basis of both qualitative and quantitative Southern blotting and DNA-sequence analysis. Most index cases who test positive for a VHL mutation have a positive family history

Table 1

Phenotypic Characteristics of Parents with VHL Mosaicism and Affected Offspring

	Family 1		Family 2	
	Index Case	Mosaic Parent	Index Case	Mosaic Parent
Sex	Female	Female	Female	Male
No. of siblings	0	…	5	…
VHL disease[a]	PC, CNS	PC, CNS, KC	CNS, K, RA, PC	CNS, PC, KC, RCC

[a] CNS = central nervous system hemangioblastomas; K = solid kidney lesion detected by CT scan; KC = renal cysts (simple renal cysts alone are not diagnostic of VHL); PC = pancreatic cysts; RCC = renal-cell carcinoma; and RA = retinal angioma.

of VHL disease; however, some individuals report no history of VHL in either parental lineage. Among 181 kindreds with VHL that were evaluated by the National Institutes of Health (NIH), 42 (23%) appeared, by family history, to be first-generation diagnoses (Glenn et al. 1999). First-generation diagnoses may result from a new mutation occurring during oogenesis or spermatogenesis in the parent. Another possibility is that the seemingly unaffected parent is mosaic for the disease because of a somatic mutation acquired during early development.

Mosaicism is defined as the presence in an individual of at least two cell lines differing in genotype and arising from a single zygote (Austin and Hall 1992). Depending on the stage of development and the cell in which the mutation occurred, the individual may be unaffected or may have clinical disease (Zlotogora 1998; Kent-First 1999). The finding of mosaicism has important implications both in counseling family members and in risk assessment for disease development in the individual with VHL mosaicism (Zlotogora 1998).

We report two detected cases of mosaicism in VHL, one in a male patient and one in a female patient. Both patients had clinical VHL disease but were negative for VHL mutations, according to testing of their peripheral blood lymphocytes (PBLs) by standard methods. Each had an offspring with known clinical VHL disease, and both offspring had a documented germline mutation in the *VHL* tumor-suppressor gene. We believe this to be the first report of mosaicism in individuals with VHL disease.

Subjects and Methods

Clinical Evaluation

Two families, each with index cases affected by VHL disease, were identified from the NIH-VHL Family Registry of patients screened under a National Cancer Institute (NCI) review board–approved protocol. The biological parents of each index case were alive and eligible for testing. In both kindreds, there was no parental history of VHL at the time of diagnosis of the index case. Subjects were counseled, and informed consent was obtained. Affection status was determined by medical examinations, conducted at the NIH Clinical Center, which included physical examinations, radiological evaluations, and laboratory testing according to methods described elsewhere (Choyke et al. 1995; Manski et al. 1997).

Mutation Analysis

Southern blot analysis and DNA-sequence analysis was performed according to methods described elsewhere (Stolle et al. 1998). FISH analysis was carried out on B-cell lymphocytes (Pack et al. 1999). Conformational sensitive gel electrophoresis (CSGE) was performed on amplified genomic or plasmid DNA, as described by Ganguly et al. (1993). PCR products of genomic DNA were cloned into a TA cloning vector (Clonetech) according to the manufacturer's instructions. Insert DNA was amplified directly from crude lysates of bacterial colonies and was mixed with the PCR product from control DNA and then was screened for mutations by CSGE and DNA sequencing. DNA was extracted from buccal swabs (Richards et al. 1993) and was amplified for 35 cycles with primers for exon 1 (Stolle et al. 1998). A 2-ml aliquot of this reaction was then reamplified for an additional 25 cycles prior to analysis.

Results

Details of clinical characteristics and mutations in the *VHL* gene in the affected offspring and mosaic parents are shown in tables 1 and 2. In family 1 (fig. 1), VHL was initially diagnosed in the daughter (III:1) at age 24 years, when she was found to have CNS hemangioblastomas. Subsequent evaluation by computed tomography (CT) of the abdomen revealed pancreatic cysts (fig. 2*A*). DNA analysis of this individual (III:1) by quantitative Southern blot revealed a deletion of one entire allele of the *VHL* gene (fig. 3, lane 3). Direct sequencing of the other allele revealed only normal sequence. Both parents of III:1 subsequently underwent full clinical evaluation at the NIH. Clinical evaluation of the mother

(II:2) revealed findings consistent with very mild VHL disease (2-mm enhancing nodule in the cerebellum, consistent with hemangioblastoma; several pancreatic cysts [fig. 2*B*]; and a solitary left-renal cyst). Quantitative Southern blot analysis of DNA extracted from PBLs of II:2 did not detect the mutation found in III:1 (fig. 3, lane 4). FISH analysis revealed the deletion of one allele of the *VHL* gene in 47% of 50 metaphases scored (Pack et al. 1999). Individual III:1 had no siblings.

In family 2 (fig. 1), the affected daughter (IV:6) was diagnosed with VHL disease at age 15 years, when she developed symptoms caused by spinal cord hemangioblastomas (fig. 2*C*). At age 20 years, she was diagnosed with multiple cystic lesions in the right kidney, a solid lesion of the left kidney, multiple pancreatic cysts, and a small peripheral retinal angioma. Approximately 1 year after IV:6 was diagnosed with VHL, her father (III:4) developed hematuria. Evaluation and subsequent treatment confirmed disease consistent with VHL, including clear-cell renal-cell carcinoma, renal cysts, pancreatic cysts, and multiple hemangioblastomas of the cerebellum and lower brainstem (fig. 2*D*). Southern blot analysis and DNA sequencing of the *VHL* gene in the father's PBLs were negative (fig. 4*A*) by the initial genetic testing, on the basis of standard methods that identified VHL mutations in 99 of 99 (Stolle et al. 1998) and in a subsequent total of 131 of 131 (Glenn et al. 1999) affected individuals. However, genetic testing of the

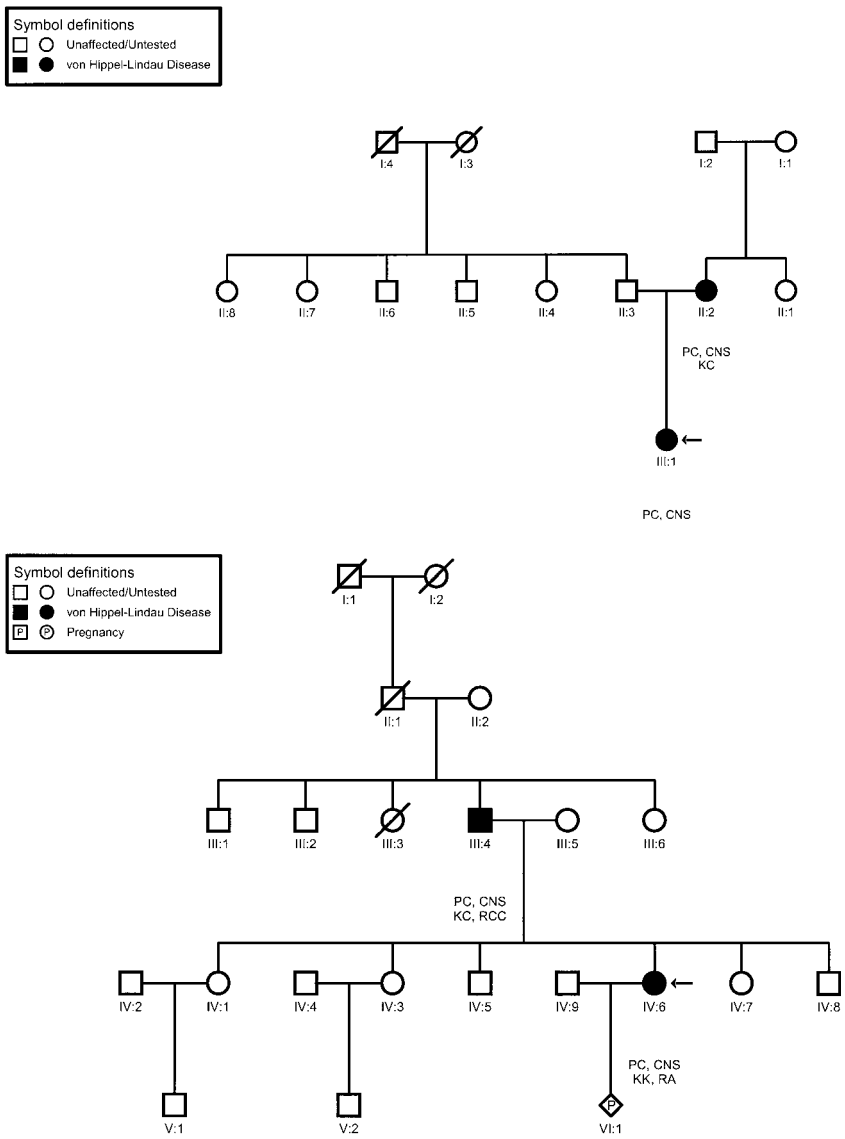

Figure 1 Pedigrees of families 1 (*top*) and 2 (*bottom*), which have VHL with parental mosaicism. Arrows indicate probands. Blackened circles and squares indicate individuals with VHL disease. For keys to disease codes, see table 1.

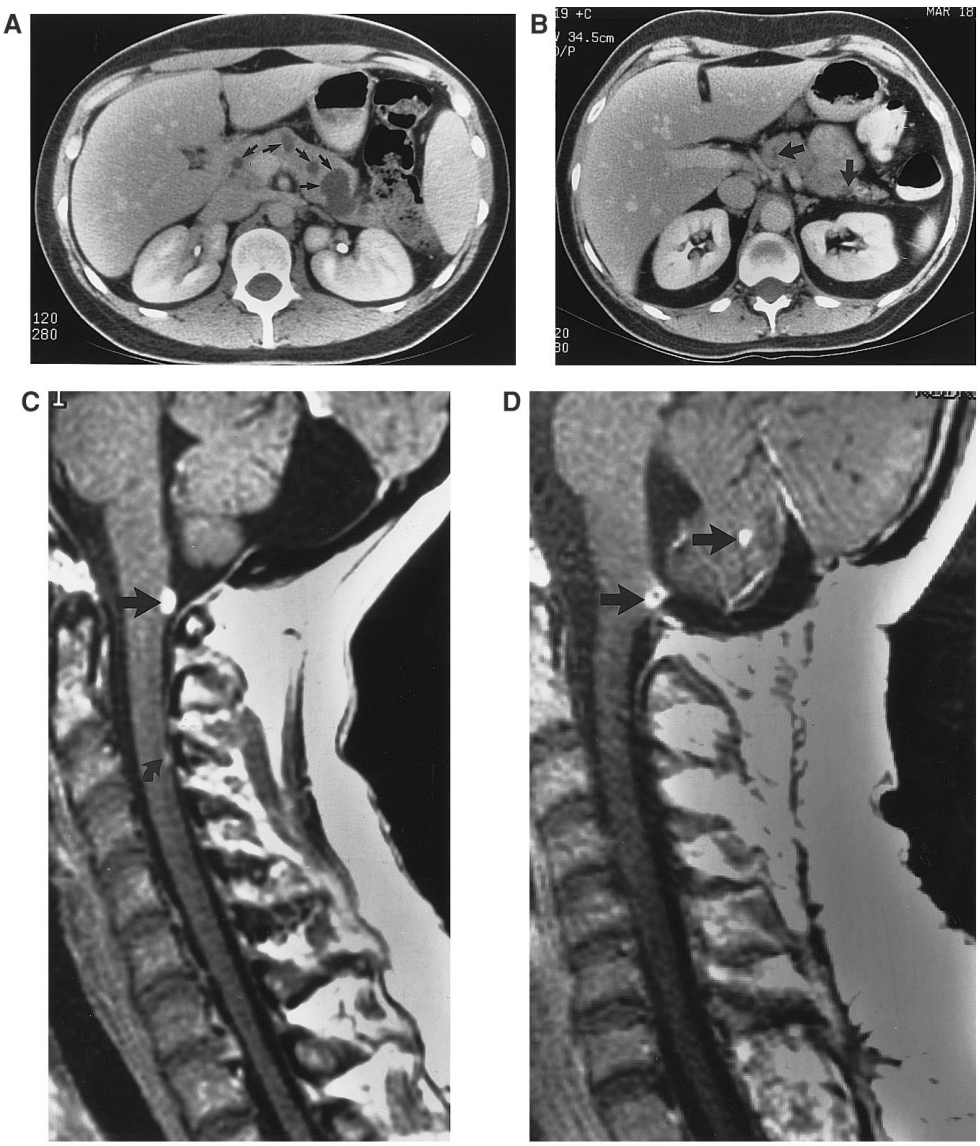

Figure 2 Radiographic images for mosaic parents and affected offspring. *A*, 27-year-old woman with VHL, from family 1 (individual III:1). Computed tomography of the abdomen demonstrates multiple cysts in the pancreas (*arrows*). *B*, 49-year-old woman, with mosaicism for VHL, from family 1 (individual II:2). Computed tomography of the abdomen demonstrates milder cystic changes within the pancreas. *C*, 23-year-old woman, with VHL, from family 2 (individual IV:6). Magnetic resonance imaging of the spine with gadolinium enhancement demonstrates hemangioblastoma (*arrow*) in the low brainstem and a tiny additional hemangioblastoma at C2-3 (*curved arrow*). *D*, 48-year-old man, with VHL mosaicism, from family 2 (individual III:4) demonstrates hemangioblastomas (*arrows*) at the same site (as IV:6) in the low brainstem and an additional lesion in the cerebellum. Additional sections (not shown) showed multiple cerebellar hemangioblastomas.

daughter's (IV:6) PBLs by direct sequencing revealed a 3-bp deletion (TTC) at nucleotides 439–441 (fig. 4*B*). After identification of the mutation in IV:6, the father's DNA was again analyzed, by application of additional methods directed specifically at the region of the 3-bp deletion. By use of CSGE, a faint shifted band was obtained with the PCR product from exon 1 (fig. 5, lane 3), suggesting the presence of at least some mutant molecules. This PCR product was cloned into a TA cloning vector and was used to transform competent bacteria. Insert DNA was amplified directly from bacterial lysates of individual colonies and was subjected to analysis by CSGE. Of the 52 colonies evaluated, 4 exhibited a gel shift. DNA-sequence analysis confirmed the presence of the delTTC mutation (fig. 4*C*). These results suggest that the father is mosaic for the mutation identified in his daughter and that the mutation was present in approximately one of seven PBLs.

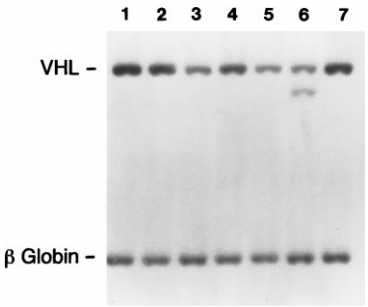

Figure 3 Quantitative Southern blot analysis, family 1. Southern blots of *Eco*RI- and *Ase*I-digested genomic DNA were hybridized to probes specific for the *VHL* gene (g7) (Latif et al. 1993) and the human beta-globin gene (Stolle et al. 1987). With equal loading of DNA, as assessed by the intensity of the beta-globin gene band, complete deletion of the *VHL* gene is apparent from the decreased intensity of the *VHL* gene band in patient samples, relative to that in unaffected controls. Samples with a partial deletion of the *VHL* gene exhibit an abnormally migrating band. Lanes 1, 2, and 7, Control DNA. Lane 3, DNA from III:1. Lane 4, DNA from II:2. Lanes 5 and 6, DNA from patients with complete or partial deletions of the *VHL* gene, respectively.

To determine the extent of mosaicism in the father (III:4), additional samples of DNA were obtained from buccal epithelial cells and cultured skin fibroblasts. The presence of the mutation in these tissues was detected by the appearance of faint bands on CSGE analysis of amplified DNA from buccal cells or cultured skin fibroblasts (fig. 5, lanes 4 and 5). These results support the conclusion that this individual is mosaic for a VHL mutation.

There were five other children (siblings of IV:6) in this family. One (IV:7) underwent clinical screening at the NIH and showed no evidence of VHL and was later shown to be negative for the 3-bp VHL deletion. The four remaining siblings have not been evaluated at the NIH; however, two siblings tested negative for the VHL mutation, by DNA-sequence analysis.

Discussion

Postzygotic mutations result in mosaicism. It is important to recognize that mosaicism is a potential cause for failure of molecular diagnosis in VHL and may manifest as a single-system disease or as a multisystem disease. Mosaicism has been described in families with inherited tumor syndromes, including retinoblastoma (Sippel et al. 1998), type 1 (Lazaro et al. 1994) and type 2 neurofibromatosis (Evans et al. 1998; Kluwe and Mautner 1998), and the two known tuberous-sclerosis genes, TSC1 (Kwiatkowska et al. 1999) and TSC2 (Yates et al. 1997). Since the gene for VHL is now known (Latif et al. 1993) and since *VHL*-gene mutations have been detected in leukocyte DNA in 100% of germline-affected individuals (Stolle et al. 1998), it may be possible to detect cases of VHL mosaicism and to screen for presymptomatic disease in at-risk individuals.

An individual mosaic for a mutation may be asymptomatic or may present with various manifestations of the disease. Although a mosaic parent tends to have less severe disease than does his or her offspring, this may not always be the case (Zlotogora 1998). In our two cases this appears to be true for family 1, in which the mosaic parent had subclinical disease found only after screening by radiographic evaluation. In the other case

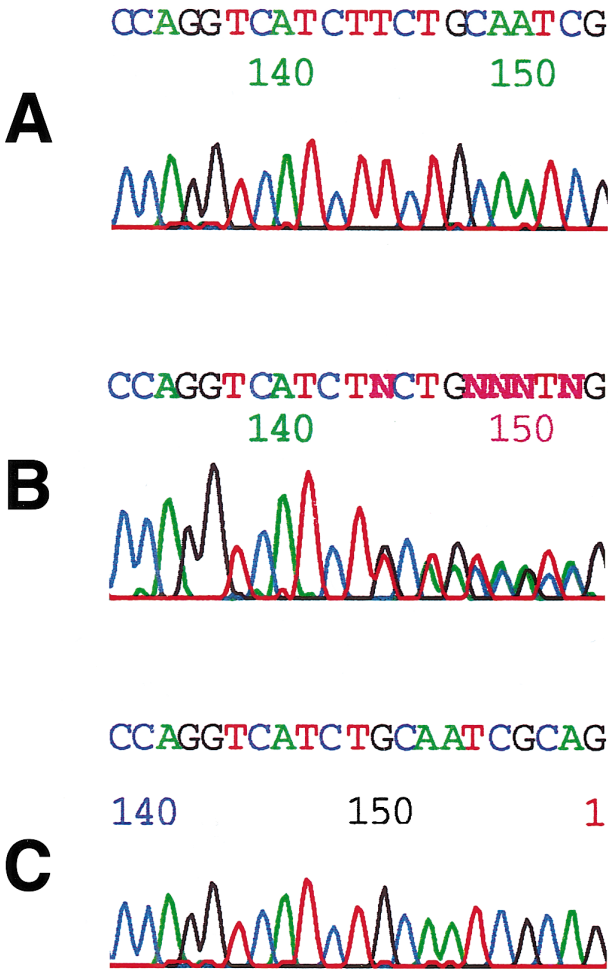

Figure 4 DNA-sequence analysis, family 2. A portion of the *VHL* gene containing exon 1 sequence was amplified as described elsewhere (Stolle et al. 1998) from either genomic DNA or plasmid DNA containing a cloned DNA insert and was subjected to DNA-sequence analysis. *A*, Sequence of genomic DNA from III:4. *B*, Sequence of genomic DNA from IV:6. *C*, Sequence of cloned DNA from III:4. Note that the sequence in *B* indicates that this patient is heterozygous for a frameshift mutation. The sequence of the cloned DNA in *C* reveals that the frameshift is due to a 3-bp (TTC) deletion in this region.

Table 2

Mutation Analysis (From Lymphocytes) of Parents with VHL Mosaicism and Affected Offspring

	Family 1		Family 2	
	Index Case	Mosaic Parent	Index Case	Mosaic Parent
Southern blot	Positive	NDM	NDM	NDM
Direct sequencing	NDM in remaining normal allele	NDM	delTTC nt 439–441	1:13 clones delTTC nt 439–441
FISH	Deletion detected	47% of cells positive for allele deletion	NA	NA
CSGE	NA	NA	NA	Gel shift detected

Note.—NA = not applicable; NDM = no detectable mutation.

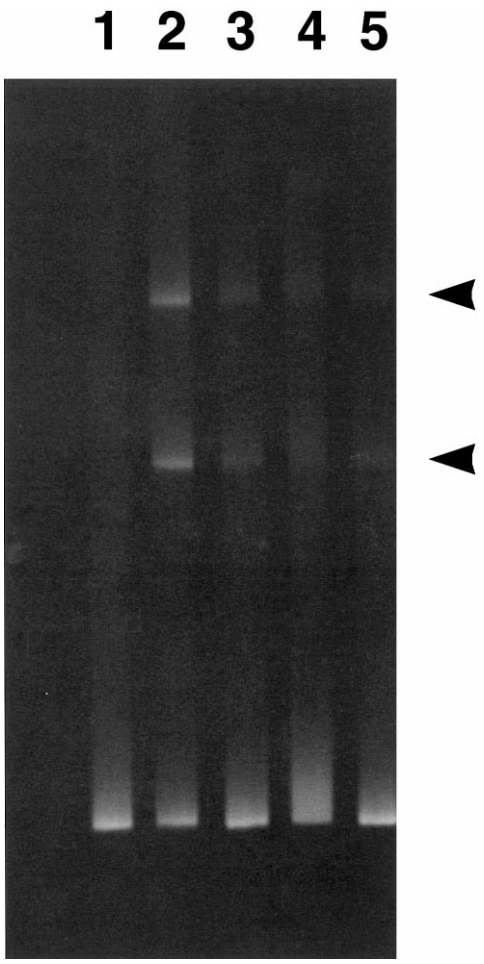

Figure 5 CSGE of samples from family 2. Genomic DNA from control (lane 1) and patient (lanes 2–5) samples was amplified with primers for exon 1. PCR products were denatured, reannealed, and analyzed by CSGE (Ganguly et al. 1993). Genomic DNA was extracted from the peripheral blood of a normal control (lane 1), peripheral blood from IV:6 (lane 2), peripheral blood from III:4 (lane 3), buccal cells from III:4 (lane 4), cultured skin fibroblasts from III:4 (lane 5). Arrows indicate position of the shifted bands.

(family 2), the VHL-mosaic parent developed advanced, symptomatic VHL disease affecting the CNS, kidney, and pancreas and was diagnosed before genetic analysis was performed. Difference in degree of disease severity, if caused by mosaicism, may reflect whether the mutation occurred in the *VHL* gene early or late in embryogenesis. Differences between family 1 and family 2, in clinical spectrum of disease expression, may be due to phenotype-genotype variations known to occur in VHL.

Parental mosaicism may account for some apparently sporadic or new cases of VHL and has important implications in the genetic counseling of families affected by VHL. An individual mosaic for a disease mutation is at increased risk for having affected offspring, despite testing negative for the mutation by routine DNA diagnostic tests. Additional molecular evaluation may be necessary to confirm the diagnosis of mosaicism. In this report, the parents with mosaicism from each kindred were negative for VHL mutations detected in their affected offspring when tested by standard Southern blot analysis and DNA sequencing of their PBLs. Tissues bearing the VHL mutation will vary from one case of mosaicism to another. When possible, additional tissue should be evaluated for mosaicism (Zlotogora 1998; Kent-First 1999). We evaluated buccal-cell DNA and fibroblasts DNA in family 2, individual III:4. For family 1, buccal cell DNA provides insufficient high-molecular-weight DNA to allow an entire allele deletion to be detected by Southern blot analysis.

Of 181 kindreds affected by VHL that were screened at the NIH, 42 (23%) included cases with VHL disease in individuals whose parents and siblings had no history of the disease at the time of their diagnosis. We have now shown 2 (4.8%) of the 42 to have parents mosaic for VHL.

This is similar to the 4% rate of somatic mosaicism reported in families affected by NF2 (Evans et al. 1998). When all living parents of affected individuals are tested, the number of mosaics among cases without a previous

family history of VHL may be >5%. It is not feasible to screen all appropriate tissue from apparently normal parents for evidence of mosaicism. Some apparently normal individuals may not choose mutation testing, because of possible economic and psychosocial implications. Parents who test negative for the VHL germline mutation in their affected offspring have a finite risk for VHL disease and may choose clinical screening. Siblings of the germline-affected offspring of a mosaic may be tested for the identified *VHL*-gene mutation.

Depending on the degree of mosaicism and the tissues affected, individuals with mosaicism are at increased risk for development of symptoms of the disease. Detecting individuals with mosaicism and performing appropriate evaluation for clinical disease may improve their survival. In addition, other at-risk family members may be evaluated for the presence of the disease mutation, prior to onset of symptoms. It is important to investigate the parents of a patient with an apparent new mutation, for the presence of mosaicism, to improve genetic counseling and medical management of these families and individuals.

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Electronic-Database Information

The accession number and URL for data in this article are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for VHL [MIM 193300])

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