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Molecular Genetic Diagnosis of von Hippel-Lindau Disease: Analysis of Five Japanese Families

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We analyzed deoxyribonucleic acids from blood samples of five Japanese von Hippel-Lindau (VHL) disease families (three familial cases, two new mutations) for the presence of VHL gene mutations by single-strand conformational polymorphism analysis and direct sequencing. Four of the five families showed germ line mutations in VHL gene, comprising 2 missense mutations, 1 deletion, and 1 splice-site mutation. Two families had VHL gene mutations at exon 1; 1 family at exon 3; and 1 family at the splice-site adjacent to exon 3. Presymptomatic patients were accurately diagnosed by these methods. However, one family did not show a VHL gene mutation in the germ line but showed a somatic mutation at exon 2 in the hemangioblastoma tissue. The consequence of the somatic mutation was a microdeletion leading to a frameshift mutation. Our study is the first report of VHL gene analyses of Japanese VHL disease families, and suggests that not only germ line mutation, but also somatic mutation can lead to development of a tumor associated with the VHL disease.

Key words: von Hippel-Lindau disease — Molecular diagnosis — Tumor suppressor gene

von Hippel-Lindau (VHL) disease is a well-known hereditary multitumor syndrome; patients develop hemangioblastomas in the central nervous system (CNS), retinal angioma, renal cell carcinomas, pheochromocytomas, and congenital pancreatic and renal cysts.[1–3] Death usually results from CNS hemangioblastomas and sometimes renal cell carcinomas.[4,5] Recently, the VHL gene, located at chromosome 3p25,[6] was isolated by positional cloning.[7] A partial 1.65-kb cDNA detected transcripts of 6 and 6.5 kb, and the cDNA encodes 852 nucleotides in 3 exons. The VHL gene has the characteristics of a classic tumor suppressor gene; loss of the wild-type allele has been demonstrated in VHL patients presenting with renal cell carcinomas and somatic mutations of the gene have been detected in sporadic renal cell carcinomas and CNS hemangioblastomas with a loss of heterozygosity.[8–10]

A total of 25 peripheral blood samples from four Japanese VHL disease families were collected at Yokohama City University (families A, B, D and E) and Nishiarai Hospital (family C). Their family trees are shown in Fig. 1. Family A consisted of five individuals including one VHL patient who had developed multiple hemangioblastomas in the cerebellum and spinal cord, retinal angioma, and giant multicystic kidneys. The patient had first developed cerebellar hemangioblastoma at 14 years of age, and had had three recurrences of cerebellar hemangioblastomas and two recurrences of spinal hemangioblastomas over the subsequent 18 years. The pathohistological study revealed characteristic capillary hemangioblastoma, showing the sheet-like appearance of tumor cells with clear cytoplasm and many capillary vessels. In this family, however, no other VHL patient was found. Family B consisted of three individuals including one VHL patient whose parents had not developed VHL disease. This patient developed brain stem hemangioblastoma and retinal angioma when he was 30 years old. Thereafter, he had no sign of recurrence for 5 years. The cases of families A and B were new mutations. Family C consisted of 10 individuals, spanning three generations, and contained three patients who had developed CNS hemangioblastomas and retinal angiomas. The eldest patient in this family (C-1) also presented with renal cell carcinoma. Family D consisted of 7 individuals including 2 patients; one had developed brain stem hemangioblastoma and retinal angioma and the other, renal cell carcinoma. Family E consisted of 11 individuals, spanning three generations, including three patients who had developed central neuroaxial hemangioblastomas and pancreas cysts (Fig. 1, Table I). DNAs from most or all members of VHL families were extracted from blood samples and from a recurrent hemangioblastoma tissue in family E, and then subjected to single-strand conformational analysis and direct sequencing. Peripheral blood from each of 25 individuals of the VHL families were collected. A hemangioblastoma tissue associated with VHL disease (family E) was obtained from a

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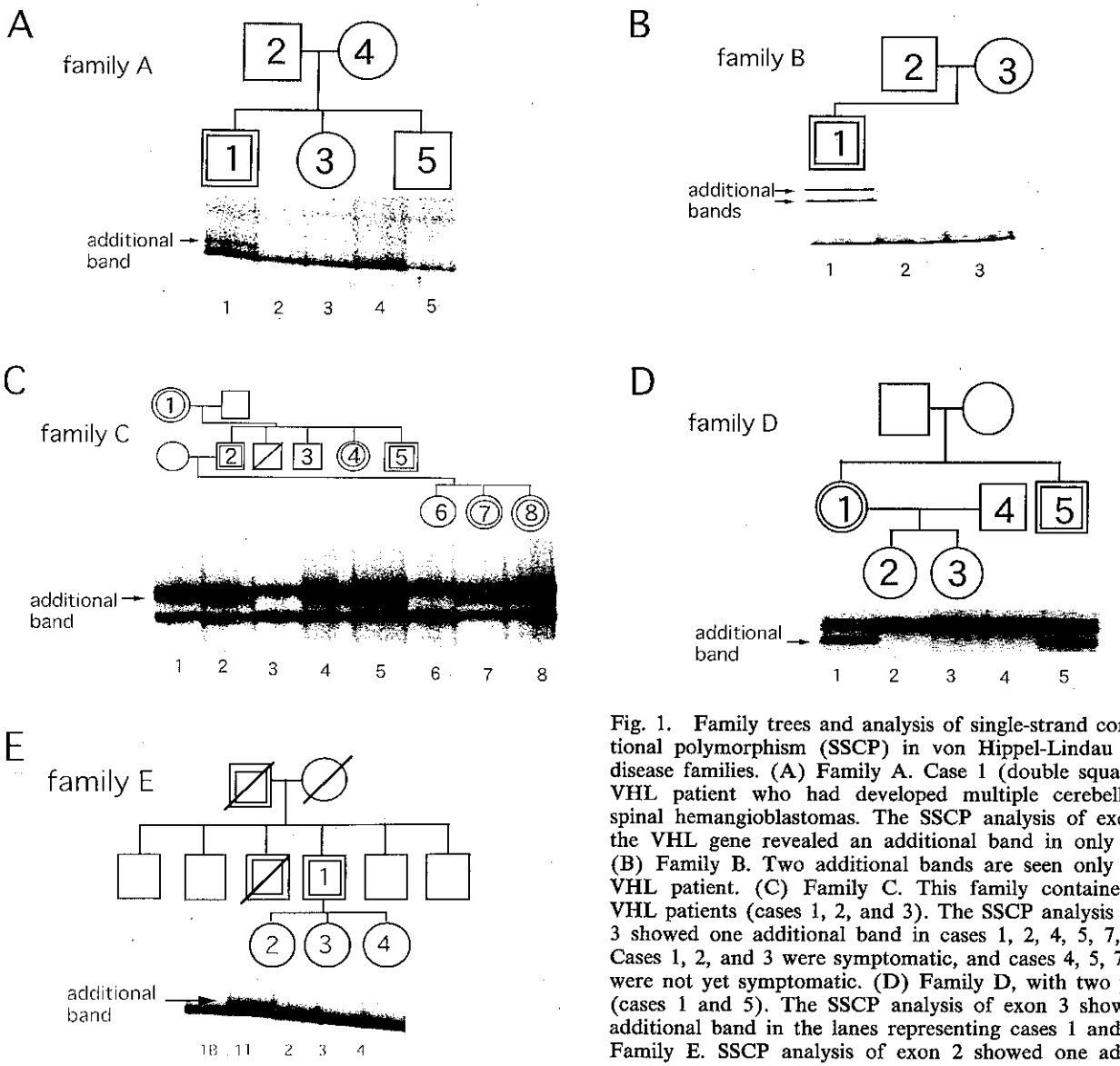

Fig. 1. Family trees and analysis of single-strand conformational polymorphism (SSCP) in von Hippel-Lindau (VHL) disease families. (A) Family A. Case 1 (double square) is a VHL patient who had developed multiple cerebellar and spinal hemangioblastomas. The SSCP analysis of exon 1 of the VHL gene revealed an additional band in only case 1. (B) Family B. Two additional bands are seen only for the VHL patient. (C) Family C. This family contained three VHL patients (cases 1, 2, and 3). The SSCP analysis of exon 3 showed one additional band in cases 1, 2, 4, 5, 7, and 8. Cases 1, 2, and 3 were symptomatic, and cases 4, 5, 7, and 8 were not yet symptomatic. (D) Family D, with two patients (cases 1 and 5). The SSCP analysis of exon 3 showed one additional band in the lanes representing cases 1 and 5. (E) Family E. SSCP analysis of exon 2 showed one additional band in hemangioblastoma tissue of case 1 (double square, VHL patient). T, tumor; B, blood.

surgical specimen. The DNA was extracted by standard procedures using proteinase K, and phenol/chloroform.

Single-strand conformational polymorphism (SSCP) analysis of the VHL tumor suppressor gene was performed using primers of exons 1, 2, and 3 (Fig. 2, Table II). Genomic DNAs were amplified by the polymerase chain reaction (PCR) technique in the following reaction mixture: 100 ng of template DNA, 1× PCR buffer, 10 pmol of each PCR primer, 500 μmol each of dGTP, dATP, dTTP and dCTP, and 0.5 units of Taq DNA polymerase (Takara, Tokyo) in a volume of 10 μl, to which was added 500 μmol of [α-^{35}S]dATP. The PCR conditions were as follows: an initial 4-min denaturation at 95°C, 40 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 2 min, and final extension at 72°C for 5 min, carried out in a thermal cycler (Perkin Elmer Cetus, Norwalk, CT). SSCP analysis was performed as described previously.[11,12] A 5 μl volume of stop-solution (10 m*M* NaOH, 95% formamide, 0.05% xylene cyanol, 0.05% bromophenol blue) was added to a 10 μl aliquot of each PCR reaction mixture, and the mixture was heated to 95°C for 5 min, then chilled on ice. Next, the

Table I. Clinical Features and Germ Line Mutations of Five von Hippel-Lindau Disease Families

Family	Case	Type of tumors	SSCP change	Sequence change	Codon change	Consequence
A	1	HBL, RA, RCy	VHL exon 1	445; A to C	149; Asn to His	Missense
	2	(−)	(−)			
	3	(−)	(−)			
	4	(−)	(−)			
	5	(−)	(−)			
B	1	HBL, RA	VHL exon 1	440–442; 3 bp deletion	147–148; Phe & Cys to Cys	1 amino acid deletion in frame
	2	(−)	(−)			
	3	(−)	(−)			
C	1	HBL, RA, RCC	VHL exon 3	677_{-1}; g to t		Splicing change
	2	HBL, RA	VHL exon 3			
	3	HBL, RA	VHL exon 3			
	4	(−)	(−)			
	5	(−)	VHL exon 3			
	6	(−)	(−)			
	7	(−)	VHL exon 3			
	8	(−)	VHL exon 3			
D	1	HBL, RA	VHL exon 3	697; T to C	233; Cys to Arg	Missense
	2	(−)	(−)			
	3	(−)	(−)			
	4	(−)	(−)			
	5	RA, RCC	VHL exon 3			
E	1	HBL[a], PC	(−)			
	2	(−)	(−)			
	3	(−)	(−)			
	4	(−)	(−)			

A, adenine; C, cytosine; g, guanine; T or t, thymine; HBL, hemangioblastoma; RA, retinal angioma; Rcy, renal cyst; RCC, renal cell carcinoma; PC, pancreas cyst.
a) A microdeletion was detected at VHL gene nucleotide 644 (codon 215) in exon 2.

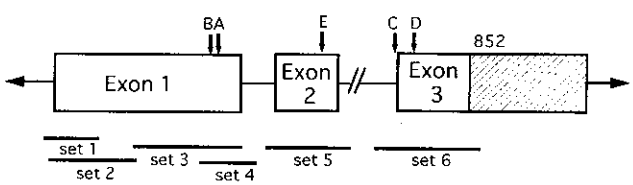

Fig. 2. VHL gene mutation sites of five Japanese VHL families and ranges of the 6 sets of primers. Arrows, germ line mutations were detected in families A, B, C, and D, and a somatic mutation alone was detected in family E. ▨, untranslated region of exon 3.

entire mixture was loaded onto non denaturing Hydrolink gels (0.5× MDE, 0.6× TBE, 10% glycerol; AT Biochemicals, Malvern, PA). The gels were run at 6 W overnight at room temperature, dried on Whatman 3MM paper and exposed to X-ray film for approximately 2 days. The further analysis of samples that were positive by SSCP analysis was performed by direct sequencing. The samples were amplified by PCR with one primer biotinylated[6] at the 5′ end in a total volume of 50 μl. Biotinylated products were collected with streptavidin-conjugated Dynabeads (Dynal AS, Oslo, Norway) followed by alkaline denaturation to retain biotinylated single-strand products.[13] These templates were subjected to the dideoxy sequencing procedure using [α-^{32}P]dATP label and Sequenase version 2.0 (U.S. Biochemicals, Cleveland, OH). The reaction products were separated on 6% denaturing polyacrylamide gels. Sequencing of both strands of genomic DNA was performed to confirm mutations.

DNA-SSCP analyses of ^{35}S-labeled PCR samples detected one or two additional bands at exon 1 in families A and B, and exon 3 in families C and D (Fig. 1). On the other hand, blood samples from family E did not show an abnormal pattern in DNA-SSCP analyses of the VHL gene, but a hemangioblastoma tissue showed one additional band at exon 2. The direct sequence analyses revealed 3 point mutations and 1 deletion (Table I). Family A showed a missense mutation at nucleotide number 445, changing adenine to cytosine. This mutation resulted in a change at codon 149 from asparagine to histidine. Family B had a deletion of thymine, cytosine, and thymine (TCT) at nucleotides 440–442. This muta-

Table II. PCR Primers for SSCP Analysis of VHL Gene

Set	Exon		Sequence of primers
1	1	Upstream	5′-AAATACAGTAACGAGTTGGCCTAGC-3′
		Downstream	5′-GTCGACCTCCGTAGTCTTCG-3′
2	1	Upstream	5′-CCTAGCCTCGCCTCCGTTACAAC-3′
		Downstream	5′-GTACTCTTCGACGCCTGCCTACC-3′
3	1	Upstream	5′-TGGTCTGGATCGCGGAGGGAAT-3′
		Downstream	5′-CCCAGCTGGGTCGGGCCTAAGCGCCGGGCCCGT-3′
4	1	Upstream	5′-CTGCCCGTATGGCTCAACTTCGA-3′
		Downstream	5′-TGCTATCGTCCCTGCTGGGT-3′
5	2	Upstream	5′-GTGGCTCTTTAACAACCTTTGC-3′
		Downstream	5′-CCTGTACTTACCACAACAACCTTATC-3′
6	3	Upstream	5′-TTCCTTGTACTGAGACCCTAGT-3′
		Downstream	5′-AGCTGAGATGAAACAGTGTAAGT-3′

tion resulted in a change at codons 147–148 from phenylalanine-cysteine to cysteine. Family C revealed a point mutation from guanine to thymine in a splice-site at nucleotide number 677_{-1} adjacent to exon 3. Family D showed a missense mutation at nucleotide number 697. The consequence of this mutation was a change at codon 233 from cysteine to arginine. A hemangioblastoma in family E showed a deletion of guanine at nucleotide 644. This mutation resulted in a frame shift with a change at codon 215 (Table I, Fig. 2).

Our analyses revealed that abnormal additional bands were detected in VHL patients: one at exon 1 in family A, and two at the same exon in family B, and at exon 3 in families C and D (Fig. 1). Family A members showed no abnormal SSCP pattern except for the one VHL patient. Family B, composed of one VHL patient and his parents, showed an abnormal SSCP pattern only in this patient, at exon 1. Family C, which contains 3 VHL patients, showed an aberrant SSCP pattern in 6 individuals including the 3 VHL patients, at exon 3. This result suggests that the 3 cases who had an abnormal SSCP pattern without any manifestation of VHL disease were presymptomatic carriers. Family D, composed of 7 individuals with two VHL patients, showed an abnormal SSCP pattern only in the two VHL patients at exon 3. Our results from SSCP and direct sequencing analyses in families A, B, C, and D were thus confirmed to reflect accurately the clinical situation in the patients and their family members. Family E, composed of 11 individuals with 3 VHL patients, did not show an abnormal SSCP pattern at any exon of the VHL gene. However, hemangioblastoma tissue from a patient of family E showed an additional band at exon 2. The consequence of this SSCP change was a microdeletion leading to a frameshift mutation.

VHL disease is a hereditary angiomatosis that develops retinal angiomas and hemangioblastomas in the cerebellum, brain stem, and spinal cord. This disorder is named after von Hippel,[14] who reported retinal angiomatosis, and Lindau, who called the combination of cerebellar hemangiomatous tumors and retinal small angiomas "angiomatosis of the CNS."[15] In this disease, vascular skin lesions and congenital benign cysts of the kidneys, pancreas, liver, and lungs are commonly encountered.[16] In addition, renal cell carcinomas and pheochromocytomas are also frequently associated with this disease. The penetrance of VHL disease is almost complete by 65 years of age, and most VHL gene carriers develop CNS hemangioblastoma.[8] It is very important to decide whether a patient is a VHL-mutated gene carrier or not, because if the former is the case, then the occurrence of CNS hemangioblastoma, retinal angioma, renal cell carcinoma, and pheochromocytoma can be predicted. The causative gene of VHL disease has been delineated in the chromosome 3p25–26 area,[6] and loss of heterozygosity in 3p has also been identified in hemangioblastomas associated with VHL disease.[17] The positional cloning for this VHL gene was successful, which made mutation analyses possible in VHL families.[7] The present study showed that germ line mutations in VHL families were detected in the latter half of exon 1 and in the first half of exon 3 in the VHL tumor suppressor gene. We also showed that two mutations (in families A and D) were missense mutations, resulting in substitutions of amino acids, that one (in family B) was a deletion of an amino acid; and that one (in family C) was a point mutation in the splice-site near exon 3. Chen *et al.* examined a large panel of Caucasian VHL families to determine the nature and distribution of mutations responsible for VHL disease.[4] Their study revealed that VHL gene mutations were found in each of the 3 coding exons: mutations were clustered at the 3′ end of exon 1 and at the 5′ end of exon 3, with a few in exon 2. In addition, the data indicated that missense mutations were predominant (61.7%) and other mutations (nonsense, deletion, insertion) occurred in 10–16% of all VHL families. On the other hand, Whaley *et al.*[18] identified germ line mutations in 39% of

61 VHL disease families and VHL gene somatic mutations in 33% of sporadic renal carcinomas. There was no distinct difference between our Japanese type and Whaley's data. The previous reports did not identify VHL gene mutations in 40–60% of VHL families. Our study revealed that family E showed no germ line mutation but had a somatic VHL gene mutation. This result suggests that the germ line mutation in family E may lie in a region which was not detected by the SSCP method. The predicted protein of the VHL gene contains an acidic pentameric repeat that has homology to the acidic repeat domain in the procyclic surface membrane protein of *Trypanosoma brucei*.[7] These data suggest that mutations in VHL gene may cause tumorigenetic changes in the cell membranes in the CNS and other organs. Recently, the VHL protein was shown to bind tightly and specially to elongins B and C, which activated transcription elongation by RNA polymerase II, and to inhibit elongin (S III) transcriptional activity.[19] This report suggests that the VHL protein may play an important role in a transcriptional regulatory network which controls tumorigenesis. VHL gene expression has been demonstrated by *in situ* mRNA hybridization in the CNS of murine embryo, in the cerebral cortex, midbrain, cerebellum, and spinal cord.[20] The widespread expression of the VHL gene in the CNS suggests that the occurrence of glial tumors may be related to mutation of the VHL gene.

Our present study revealed DNA-SSCP analysis of the VHL gene to be useful for genetic screening of VHL families. In addition to the disclosure of asymptomatic VHL-mutated gene carriers, our analysis confirmed the feasibility of prenatal diagnosis of VHL disease. Asymptomatic VHL-mutated gene carriers may thus be given the opportunity to undergo early diagnosis and treatment of CNS hemangioblastoma and/or renal cell carcinoma. The VHL-mutated gene carriers should undergo regular screening tests every 6 months or 1 year by computerized tomography, magnetic resonance imaging, and abdominal ultrasonic detection. However, as in the case of family E, the possibility of false-negative diagnosis using DNA-SSCP analysis should be considered. Somatic mutations and loss of heterozygosity in VHL tumor suppressor gene were frequently detected in sporadic hemangioblastomas and renal cell carcinomas.[9, 10] These reports suggested that inactivation of two alleles in the VHL gene was essential in the development of these tumors. Therefore, the VHL gene would appear to have the characteristics of a recessive tumor suppressor gene, which is consistent with Knudson's two-hit model of tumorigenesis.[21]

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REFERENCES

1) Go, R. C. P., Lamiell, J. M., Hsia, Y. E., Yuen, J. M.-M. and Paik, Y. Segregation and linkage analyses of von Hippel Lindau disease among 220 descendants from one kindred. *Am. J. Hum. Genet.*, **36**, 131–142 (1984).
2) Huson, S. M., Harper, P. S., Hourihan, M. D., Cole, G., Weeks, R. D. and Compston, D. A. S. Cerebellar hemangioblastoma and von Hippel Lindau disease. *Brain*, **109**, 1297–1310 (1986).
3) Memon, K. L. and Rosen, S. W. Lindau's disease. *Am. J. Med.*, **36**, 595–617 (1964).
4) Chen, F., Kishida, T., Yao, M., Hustad, T., Glavac, D., Dean, M., Gnarra, J. R., Orcutt, M. L., Duh, F. M., Glenn, G., Green, J., Hsia, Y. E., Lamiell, J., Li, H., Wei, M. H., Schmidt, L., Tory, K., Kuzmin, I., Stackhouse, T., Latif, F., Linehan, W. M., Lehman, M. and Zwar, B. Germ line mutations in the von Hippel-Lindau disease tumor disease tumor suppressor gene, correlation with phenotype. *Hum. Mutat.*, **5**, 66–75 (1995).
5) Horton, W. A., Wong, V. and Eldridge, R. von Hippel-Lindau disease. Clinical and pathological manifestations in nine families with 50 affected members. *Arch. Intern. Med.*, **136**, 769–777 (1976).
6) Seizinger, B. R., Rouleau, G. A., Ozelius, L. J., Lane, A. H., Farmer, G. E., Lamiell, J. M., Haines, J., Yuen, J. W. M., Collins, D., Majoor-Krakauer, D., Bonner, T., Matthew, C., Rubenstein, A., Halperin, J., McConkie-Rossel, A., Green, J. S., Troffatter, J. A., Ponder, B. A., Eierman, L., Bowner, T., Schmike, R., Oostra, B., Aronin, N., Smith, D. I., Drabkin, H., Wazari, M. W., Hobbs, W. J., Martuza, R. L., Conneally, P. M., Hsia, Y. E. and Gusella, J. F. von Hippel-Lindau disease maps to the region of chromosome 3 associated with renal cell carcinoma. *Nature*, **332**, 268–269 (1988).
7) Latif, F., Gnarra, J., Tory, K., Grarra, J., Yao, M., Duh, F.-M., Orcutt, M. L., Stackhouse, T., Kuzmin, I., Modi, W., Geil, L., Schmidt, L., Zhou, F., Li, H., Wei, M. H., Chen, F., Glenn, G., Choyke, P., Walther, M. M., Weng, Y., Duan, D. R., Dean, M., Glavac, D., Richards, F. M., Crossey, P. A., Ferguson-Smith, M. A., Paslier, D. L., Chumkov, I., Cohen, D., Chinault, C., Mahler, E. R.,

Linehan, W. M., Zwar, B. and Lerman, M. I. Identification of the von Hippel-Lindau disease tumor suppressor gene. *Science,* **260,** 1317–1320 (1993).

8) Gnarra, J. R., Tory, K., Weng, Y., Schmidt, L., Wei, M. W., Li, H., Latif, F., Liu, S., Chen, F., Duh, F.-M., Lubensky, I., Duan, D.-S. R., Florence, C., Pozzatia, R., Walther, M. M., Bander, N. H., Grossman, H. B., Bauch, H., Brooks, J. D., Isaacs, W. B., Lerman, M. I., Zwar, B. and Linehan, W. M. Mutations of the VHL tumor suppressor gene in renal carcinoma. *Nat. Genet.,* **7,** 85–90 (1994).
9) Kanno, H., Kondo, K., Ito, S., Yamamoto, I., Fujii, S., Torigoe, S., Sakai, N., Hosaka, M., Shuin, T. and Yao, M. Somatic mutations of the von Hippel-Lindau tumor suppressor gene in sporadic central nervous system hemangioblastomas. *Cancer Res.,* **54,** 4845–4847 (1994).
10) Shuin, T., Kondo, K., Torigoe, S., Kishida, T., Kubota, Y., Hosaka, M., Nagashima, Y., Kitamura, K., Latif, F., Zbar, B. and Lerman, M. I. Frequent somatic mutation and loss of heterozygosity of the von Hippel-Lindau tumor suppressor gene in primary human renal cell carcinomas. *Cancer Res.,* **54,** 2852–2854 (1994).
11) Glavac, D. and Dean, M. Optimization of the single-strand conformational polymorphism (SSCP) technique for detection of point mutations. *Hum. Mutat.,* **2,** 404–414 (1993).
12) Orita, M., Suzuki, T., Sekiya, T. and Hayashi, K. Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. *Genomics,* **5,** 874–879 (1989).
13) Hultman, T., Stahl, S., Hornes, E. and Uhlen, M. Direct solid phase sequencing of genomic and plasmid DNA using magnetic beads as solid support. *Nucleic Acids Res.,* **17,** 4937–4945 (1989).
14) Von Hippel, E. Die anatomische Grundlage ber von mir beschriebenen "sehr selten Erkrankung der Netzhaut." *Arch. Ophthalmol.,* **79,** 350–377 (1911).
15) Lindau, A. Cysts in the cerebellum; structure, pathogenesis and relations to angiomatosis of the retina. *Acta Pathol. Microbiol. Scand.* **1** (Suppl.), 1–128 (1926).
16) Russel, D. S. and Rubinstein, L. J. "Von Hippel-Lindau Disease. Pathology of Tumors of the Nervous System," 5th Ed., pp. 784–787 (1989). Edward Arnold, London.
17) Tory, K., Brauch, H., Linehan, M., Barba, D., Oldfield, E., Filling-Katz, M., Seizinger, B., Nakamura, Y., White, R., Marshall, F. F., Lerman, M. I. and Zwar, B. Specific genetic change in tumors associated with von Hippel Lindau disease. *J. Natl. Cancer Inst.,* **81,** 1097–1101 (1989).
18) Whaley, J. M., Naglich, J., Gelbert, L., Hsia, Y. E., Lamiell, J. M., Green, J. S., Collins, D., Neumann, H. P. H., Laidlaw, J., Li, F. P., Klein-Szanto, A. J. P., Seizinger, B. R. and Kley, N. Germ-line mutations in the von Hippel-Lindau tumor suppressor gene are similar to somatic von Hippel-Lindau aberrations in sporadic renal cell carcinomas. *Am. J. Hum. Genet.,* **55,** 1092–1102 (1994).
19) Duan, D. R., Pause, A., Burgess, W. H., Aso, T., Chen, D. Y. T., Garrett, K. P., Conaway, R. C., Conaway, J. W., Linehan, W. M. and Klauser, R. D. Inhibition of transcription elongation by the VHL tumor suppressor protein. *Science,* **269,** 1402–1406 (1995).
20) Kassler, P. M., Vasavada, S. P., Rackley, R. R., Stackhouse, T., Duh, F.-M., Latif, F., Lerman, M. I., Zwar, B. and Williams, B. R. G. Expression of the von Hippel-Lindau tumor suppressor gene in human fetal kidney and during mouse embryogenesis. *Mol. Med.,* **1,** 457–466 (1995).
21) Knudson, A. G. Mutation and cancer: statistical study of retinoblastoma. *Proc. Natl. Acad. Sci. USA,* **68,** 1297–1310 (1971).