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Germ-line Mutation Analysis in Patients with von Hippel-Lindau Disease in Japan: An Extended Study of 77 Families

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We have previously reported on the analysis of germ-line mutations in Japanese von Hippel-Lindau disease (VHL) patients and found mutations in 26 families. We have now extended these studies to include an additional 41 VHL families. Germ-line mutation of the *VHL* gene was screened by DNA-SSCP, direct sequencing, and Southern blot analysis. To summarize all of the data we have studied in this and our previous report, germ-line mutations have been detected in 55 of 77 (73%) (type 1: 41/62 (66%) and type 2: 14/15 (93%)) families. We found similarities in the nature of germ-line mutations including mutational incidence, location, and DNA substitution patterns between Japanese and Western VHL. These similarities may reflect the predominance of endogenous mutational processes. We also found several interesting characteristics in Japanese VHL. Twenty of 41 (49%) intragenic mutations were unique and not reported in the Western VHL. Four mutations (Arg113Stop, Gln132Stop, Leu158Val, and Cys162Tyr) previously characterized as type 1 mutations were identified in the type 2 (with pheochromocytoma) Japanese families. Genotype-phenotype correlation study suggested non-missense mutations predicted to result in the loss of VHL function were associated with the occurrence of renal cell carcinoma, as in sporadic tumors. Our data add to the diversity of VHL germ-line mutations and provide a better understanding of VHL disease in terms of both clinical management and molecular pathogenesis.

Key words: *VHL* gene — Tumor suppressor — Mutation — Germ-line — Polymorphism

von Hippel-Lindau disease (VHL) (MM#193300) is a rare, dominantly inherited disorder characterized by a predisposition to develop multiple neoplastic lesions including central nervous system (CNS) hemangioblastomas, retinal angiomas, pancreatic tumors, renal cell carcinomas (RCCs), and pheochromocytomas.[1)] In 1993, the *VHL* gene, located at chromosome 3p25, was isolated by a positional cloning approach and shown to be mutated in the germ-line of patients with VHL, as well as in sporadic tumors including clear-cell renal carcinomas, CNS hemangioblastomas, epididymal and pancreatic cystadenomas.[2–8)] Mutational analysis in both VHL patients and sporadic tumors demonstrated that the *VHL* gene acts as a classical tumor-suppressor inactivated by a "two mutation" mechanism.[9, 10)]

Several proteins interacting with *VHL* gene product (pVHL), including Elongin B/C-Cul2-Rbx1 complex, VBP-1, Sp1, protein kinase C (PKC), fibronectin, and hypoxia inducible factor (HIF), have been identified, and pVHL appears to have multiple functions, although the mechanisms involved are not well understood.[11–18)]

The identification of *VHL* gene allowed accurate presymptomatic diagnosis by mutation detection, and studying genotype-phenotype correlation will improve clinical management of the VHL kindreds.[8, 19)] Moreover, the identification of the spectrum of *VHL* gene mutations is of great interest in the study of the biological properties of pVHL.

To clarify the nature of germ-line VHL mutations in Japanese patients, and to examine the genotype-phenotype correlation, we have previously reported an analysis of germ-line mutations in Japanese VHL patients, in which we found mutations in 26 families.[20)] We have now extended these studies to include an additional 41 families. This series, together with our previous report, forms the largest and most comprehensive series of VHL families from Japanese-Asian populations studied to date. Our data will allow improvements in the clinical management of VHL families as well as providing a better understanding of the VHL tumor suppressor gene.

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MATERIALS AND METHODS

VHL patients A diagnosis of VHL disease was made on the basis of conventional criteria.[1)] A total of 77 unrelated VHL patients were selected on the basis of availability and their willingness to donate blood samples. Thirty-six of them (family 1 to 46) were previously studied and reported.[20)] The other 41 families (family 47 to 111) were newly analyzed in this study. When possible, we tracked the genetic alterations through pedigree trees and performed predicting tests in individuals at risk. Informed consent was obtained from all patients and family members.

DNA extraction DNA was extracted from peripheral blood or from Epstein-Barr virus-transformed lymphoblastoid cell lines by using standard methods.[21)]

Single strand conformation polymorphism (SSCP) and sequencing analysis DNA-SSCP analysis for exons 2 and 3 of the *VHL* gene was basically the same in our previous report.[4)] The polymerase chain reaction (PCR) primers previously used for exon 1 analysis sometimes amplified a false positive product mainly due to the relatively high incidence of C, G nucleotides (almost 70%) in this region. We therefore redesigned PCR primers for exon 1 and performed direct sequencing without SSCP screening to improve the frequency of mutational detection in the current study. PCR primers for exon 1 amplification were as follows: forward, 5′-TGG TCT GGA TCG CGG AGG GAA T-3′; and reverse, 5′-GAC CGT GCT ATC GTC CCT GC-3′. Sequencing reaction was performed directly from PCR-amplified DNA, by using the BigDye terminator cycle sequencing FS ready reaction kit (Perkin-Elmer Japan, Chiba), on an automated sequencer (ABI PRISM 310 Genetic Analyzer; Perkin-Elmer Japan), with the same primers as had been used for SSCP of exons 2 and 3, and the following primer for exon 1: 5′-TGC TAT CGT CCC TGC TGG GT-3′. In addition, we performed direct sequencing of exons 2 and 3 in the cases with negative SSCP and Southern blot analyses to improve the mutation detection.

Southern blot analysis Southern blot analysis was performed according to the previous reports.[2, 20)] A 5 μg aliquot of DNA was digested with restriction enzymes (*Eco*RI, *Hin*dIII or *Bgl*II) (New England Biolabs, Beverly, MA) according to the manufacturer's protocol, then electrophoresed and transferred to nylon membranes. Nylon membranes were hybridized with ^{32}P-labeled VHL cDNA (g7-11) and autoradiogramed by standard methods.[2, 21)]

Genotype-phenotype correlation studies For each phenotype of interest (CNS hemangioblastoma, retinal angioma, pancreatic cyst or tumor, renal cell carcinoma, and pheochromocytoma), the proportions affected within different mutation classes were compared by means of a standard χ^2 test. A total of 103 VHL patients from 55 mutation-positive families were examined. Germ-line mutations were grouped into 2 classes; missense mutations that resulted in single amino acid substitution in the whole pVHL, and non-missense mutations predicted to result in immature C-terminal truncation or gross alteration of pVHL.

RESULTS

We newly studied 41 VHL families for germ-line mutations by SSCP, direct sequencing, and Southern blot analysis, and detected germ-line mutations in 27 families. In addition, we re-examined mutation-negative cases in our previous study by means of direct sequencing of the entire coding region and intron junctions. As a result, we identified 2 families (family 28 and 38), each of which had a germ-line intragenic mutation in exon 1 (Table I).

In the present study and our previous report,[20)] we have analyzed a total of 77 Japanese VHL families and found germ-line mutations in 55 (71%) cases (Tables I and II). Of 55 germ-line mutations identified, 52 mutations were intragenic small alterations identified by SSCP and/or sequencing and 3 were genomic rearrangements probably due to large deletions detected by Southern blot analysis (Table III). The 52 intragenic mutations consisted of 31 missense, 4 splice-site, 3 insertion, 2 nonsense, and 2 micro-deletion mutations, and occurred between codons 72 and 198 of the gene (Table III, Fig. 1). Thus, missense mutations that resulted in a single amino acid change of the pVHL accounted for more than half (31/55: 56%) of the mutations identified. Of 52 intragenic germ-line alterations, we found 41 independent mutations. Seven intragenic mutations were observed more than once. Twenty of 41 (49%) independent mutations were found only in our Japanese series. The other 21 mutations were identical to those described previously in Western VHL (Table I).

VHL families can be classified into 2 subtypes on the basis of disease manifestations.[8)] Of 77 families analyzed, 62 (81%) families were categorized as VHL type 1 (without pheochromocytoma), and 15 (19%) as type 2 (with pheochromocytoma) (Table II). Germ-line mutations were detected in 41/62 (66%) of the type 1, and 14/15 (93%) of the type 2 families (Table II). Forty-one germ-line mutations in the type 1 family consisted of 20 missense, 12 micro-deletion, 4 splice-site, 2 insertion, and 3 Southern mutations (Tables I and III). Fourteen type 2 mutations consisted of 11 missense, 2 nonsense, and 1 insertion mutations. Most (11/14: 79%) were predicted to result in a single amino acid change of the pVHL, and the codon 167 missense mutation was found in 6 independent families. Of note, 4 mutations (Arg113Stop, Gln132Stop, Leu158Val, and Cys162Tyr) previously characterized as VHL type 1 mutations were observed in the type 2 (with pheochromocytoma) Japanese families (Table I).

Table I. Summary of VHL Germ-line Mutations Identified in VHL Patients from Japan

Family ID# [a]	Exon	Mutation [b]	Codon [c]	Consequence	CpG site	New site [d]	Type	Clinical features [e]				
								CNS	RA	PCT	RCC	Pheo
98	1	428 del 1-bp	72	frameshift			1	1/1	1/1	0/1	0/1	
2	1	437 del 3-bp	76	del Phe			1	1/1	0/1	1/1	1/1	
80	1	437 del 3-bp	76	del Phe			1	1/1	0/1	1/1	1/1	
16	1	443 del 1-bp	77	frameshift		yes	1	1/1	0/1	0/1	1/1	
1	1	445 A→C	78	Asn to His	no		1	1/1	1/1	0/1	0/1	
9	1	446 A→C	78	Asn to Ser	no		1	3/3	3/3	2/3	0/3	
22	1	452 del 1-bp	80	frameshift		yes	1	1/1	1/1	0/1	1/1	
37	1	454 C→T	81	Pro to Ser	yes		1	1/1	0/1	0/1	1/1	
42	1	469 C→T	86	Pro to Ser	no		1	1/1	1/1	1/1	1/1	
64	1	469 C→T	86	Pro to Ser	no		1	1/1	0/1	0/1	0/1	
39	1	470 C→T	86	Pro to Leu	no		1	0/1	1/1	0/1	0/1	
8	1	476 G→C	88	Trp to Ser	no	yes	1	0/1	0/1	1/1	1/1	
58	1	482 A→T	90	Asn to Ile	no	yes	1	0/1	0/1	0/1	1/1	
81	1	482 A→T	90	Asn to Ile	no	yes	1	1/1	0/1	1/1	1/1	
65	1	491 G→A	93	Gly to Asp	no	yes	1	1/1	0/1	1/1	1/1	
78	1	491 G→A	93	Gly to Asp	no	yes	1	2/2	1/2	2/2	1/2	
90	1	498 del 3-bp	96	del Gln		yes	1	1/2	0/2	2/2	1/2	
28	1	500 A→C	96	Gln to Pro	no		1	1/1	0/1	1/1	1/1	
25	1	501 del 3-bp	96–97	Gln-Pro to His		yes	1	0/1	1/1	1/1	1/1	
48	1	501 del 3-bp	96–97	Gln-Pro to His		yes	1	1/1	1/1	1/1	1/1	
38	1	542 ins 2-bp	109	frameshift		yes	1	0/1	0/1	0/1	1/1	
61	1	546 C→A	111	Ser to Arg	no	yes	1	4/4	2/4	2/4	1/4	
74	1	547 T→A	112	Tyr to Asn	no	yes	1	2/2	2/2	2/2	0/2	
83	1	564 G→T	117	Trp to Cys	no		1	1/1	0/1	1/1	0/1	
46	2	566 T→C	118	Leu to Pro	no		1	0/2	0/2	1/2	2/2	
14	2	571 ins 2-bp	120	frameshift		yes	1	0/2	0/2	0/2	2/2	
26	2	642 del10-bp	143	frameshift		yes	1	1/1	1/1	0/1	1/1	
32	2	676 G→A	155	splice mutat	no	yes	1	5/9	1/9	2/9	9/9	
107	2	676+1 G→A	155	splice mutat	no		1	1/1	1/1	1/1	1/1	
3	3	677−1 G→T	155	splice mutat	no		1	7/7	3/7	1/7	3/7	
68	3	677−1 G→A	155	splice mutat	no		1	0/1	1/1	1/1	1/1	
4	3	697 T→C	162	Cys to Arg	no		1	1/6	2/6	1/6	3/6	
11	3	698 G→A	162	Cys to Tyr	no	yes	1	4/4	0/4	0/4	0/4	
5	3	713 G→A	167	Arg to Gln	yes		1	2/2	2/2	0/2	1/2	
63	3	716 del1-bp	168	frameshift		yes	1	2/3	0/3	1/3	3/3	
105	3	729 del 1-bp	172	frameshift		yes	1	1/1	0/1	1/1	1/1	
34	3	746 T→C	178	Leu to Pro	no		1	1/1	1/1	0/1	1/1	
67	3	805 del 2-bp	198	frameshft		yes	1	1/1	1/1	1/1	1/1	
20		Southern					1	1/3	0/3	1/3	2/3	
23		Southern					1	1/1	0/1	0/1	1/1	
30		Southern					1	1/1	1/1	1/1	1/1	
106	1	506 A→G	98	Tyr to Cys	no		2	0/1	0/1	0/1	1/1	1/1
47	1	550 C→T	113	Arg to Stop	yes	*	2	1/1	0/1	1/1	1/1	1/1
87	2	605 A→G	131	Arg to Ser	no	yes	2	1/2	2/2	1/2	0/2	2/2
108	2	607 C→T	132	Glu to Stop	no	*	2	1/1	1/1	0/1	0/1	1/1
45	3	683 C→T	157	Thr to Ile	no		2	0/2	0/2	0/2	2/2	1/2
69	3	685 C→G	158	Leu to Val	no	*	2	2/2	0/2	2/2	1/2	1/2
104	3	698 G→A	162	Cys to Tyr	no	*	2	0/1	1/1	1/1	1/1	1/1
19	3	712 C→T	167	Arg to Trp	yes		2	1/1	1/1	0/1	1/1	1/1
31	3	712 C→T	167	Arg to Trp	yes		2	2/2	0/2	0/2	1/2	2/2
66	3	712 C→T	167	Arg to Trp	yes		2	1/2	0/2	0/2	1/2	1/2
102	3	712 C→T	167	Arg to Trp	yes		2	1/2	2/2	0/2	0/2	2/2
77	3	713 G→A	167	Arg to Gln	yes		2	1/3	1/3	2/3	0/3	1/3
79	3	713 G→A	167	Arg to Gln	yes		2	0/1	1/1	0/1	0/1	1/1
7	3	776 ins 20-bp	188	frameshift		yes	2	1/4	4/4	2/4	0/4	3/4

a) Families 1 to 46 were previously studied and reported.[20] Families 47 to 111 were newly analyzed in this study.
b) Nucleotides numbered according to sequence numbering by Latif *et al.*[2]
c) Codon 1 was located at nucleotides 214.
d) * Previously reported as VHL type 1 mutations.
e) CNS, hemangioblastoma of CNS; RA, retinal angioma; PCT, pancreatic cyst or tumor; RCC, renal cell carcinoma; Pheo, pheochromocytoma.

Table II. Summary of Germ-line Mutations in VHL Families: Japan vs. Europe and North America

VHL type	Country[a)]	Number of families	Number of families with mutation (%)	
			Positive	Negative
1	Jp	62	41 (67)	21 (33)
	EA	295	183 (63)	109 (37)
2	Jp	15	14 (93)	1 (7)
	EA	74	62 (84)	12 (16)
Unknown	EA	55	26 (47)	29 (53)
Total	Jp	77	55 (71)	22 (29)
	EA	424	274 (65)	150 (35)

a) Jp, current series from Japan; EA, excludes Japanese VHL data from Zbar *et al*.,[8)] and represents summarized and revised data of 7 series from Europe and North America.

Table III. Comparison of the VHL Germ-line Mutational Pattern; Japan vs. Europe and North America

VHL type	Country[a)]	Number of families	Number of mutations[b)]						
			Missense	Nonsense	Micro-deletion	Insertion	Splice	Southern	Unidentified
1	Jp	62	20	0 *	12	2	4	3 **	21
	EA	295	61	29 *	26	12	8	50 **	109
2	Jp	15	11	2	0	1	0	0	1
	EA	74	58	1	0	1	0	2	12
Unknown	EA	55	10	5	5	2	1	0	29
Total	Jp	77	31	2	2	3	4	3	22
	EA	424	129	35	35	15	9	52	150

a) Abbreviations are as defined in Table II.
b) Nonsense (∗) and Southern mutations (∗∗) in the type 1 family are significantly lower in Japan (Fisher's exact test: P=0.0031 and 0.0086, respectively).

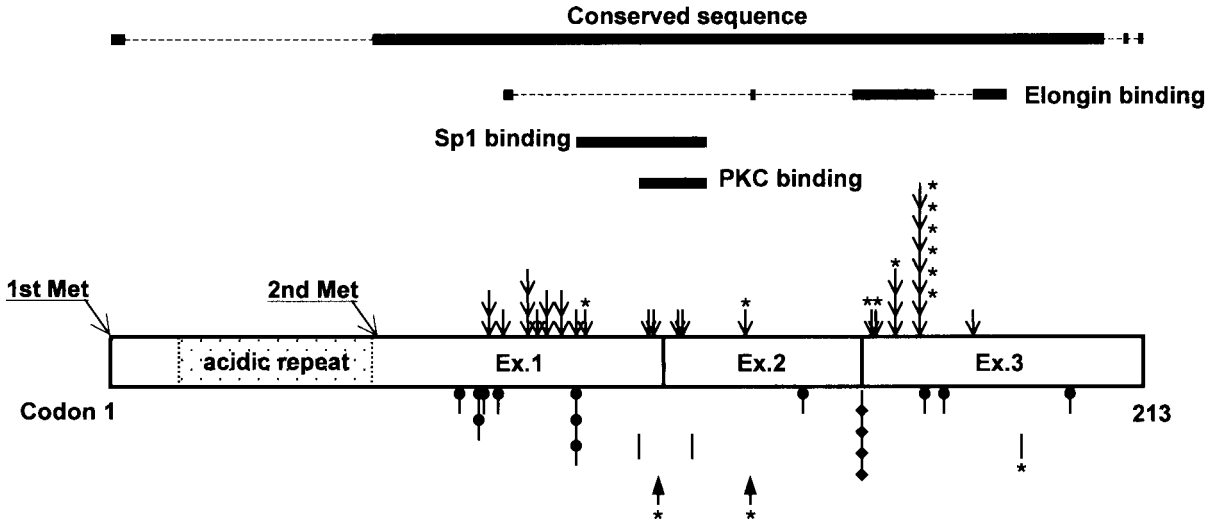

Fig. 1. The distribution of germ-line intragenic mutations in Japanese VHL. The three coding exons are indicated by boxes, the acidic pentameric repeat in exon 1 is shown as a shaded box, the first and 2nd methionines (amino acid positions 1 and 54, respectively) indicate potential translation start sites. Horizontal bars show conserved amino acid sequences and binding domains. ∗ Mutations found in the type 2 VHL families. ↓ Missence, ● deletion, | insertion, ▲ nonsense, ◆ splice site.

Table IV. Genotype-phenotype correlation in the VHL

Type of mutation	Number of patients	Number of patients with disease phenotype[a] (%)				
		CNS	RA	PCT	RCC	Pheo[b]
Missense	56	36 (64)	24 (43)	22 (39)	24 (43)	14/19 (74)
Non-missense	47	31 (66)	18 (38)	19 (40)	35 (74) *	5/6 (83)
Total	103	67 (65)	42 (41)	41 (40)	59 (57)	19/25 (76)

a) Abbreviations are as defined in Table I. Each phenotype was tested by Fisher's exact test for association with VHL mutational types. RCC (*) was significantly more common in non-missense mutations (P=0.0024).
b) Excludes type 1 families.

Table V. Nucleotide Substitutions in Germ-line VHL Mutations: Japan vs. Europe and North America

Country[a] (*n*)	Number of nucleotide substitutions (%)					
	G:C→			A:T→		
	A:T [at CpG]	T:A	C:G	G:C	T:A	C:G
Jp (37)	21 (57) [9 (24)]	3 (8)	2 (5)	5 (14)	3 (8)	3 (8)
EA (172)	74 (43) [53 (31)]	22 (13)	23 (13)	29 (17)	10 (6)	14 (8)

a) Abbreviations are as defined in Table II.

We examined genotype-phenotype correlation in our VHL family panel. When we classified the mutations into 2 types, missense mutations that change an amino acid in the pVHL and non-missense mutations that cause truncation or gross disruption of the pVHL, there was no correlation with CNS hemangioblastoma, retinal angioma, pancreas cyst or tumor, and pheochromocytoma in the type 2 family. On the other hand, renal cell carcinoma occurred predominantly in the non-missense mutation group (χ^2: P=0.0024) (Table IV).

We characterized a total of 37 germ-line single base-pair substitutions of the *VHL* gene. G:C to A:T transitions were the most frequent (21/37: 57%) changes, followed by A:T to G:C transitions (14%), and 43% of the G:C to A:T transitions (9/21 cases) were found to occur within CpG dinucleotides (Table V).

During the mutational analysis of the *VHL* gene, we identified 2 rare base changes. Two individuals exhibited A-to-G change at nucleotide 627, and 1 individual showed A-to-T change at nucleotide 675, in both cases, no amino acid was changed. We therefore regarded these DNA substitutions as polymorphisms.

DISCUSSION

In the current analysis and our previous study, we examined a total of 77 Japanese VHL families, including 62 type 1 and 15 type 2 families, for germ-line mutations of the *VHL* gene. Germ-line mutations in VHL kindreds have been studied by several different groups, and so far, mutations in more than 400 VHL families from Europe and North America have been characterized and reported by Zbar and colleagues.[8] The mutational detecting methods in our study, including SSCP, sequencing, and Southern analysis, were almost the same as those used to examine Western VHL.[8] The main characteristics of mutations in Japanese VHL were comparable to those found in the Western VHL.

We identified 52 intragenic mutations, of which all were located between codons 72 and 198. So far, intragenic mutations found both in germ-line and sporadic tumors are located exclusively in the highly conserved amino acid sequence region of the human *VHL* gene, and no mutation has been found in the non-conserved regions of the N-terminus upstream of the 2nd methionine or C-terminus.[3–8, 22–24] Our data are consistent with this finding. Recent studies about VHL protein suggested that, although 2 different-sized products (about 30 and 19 kDa) are translated from human *VHL* gene, the smaller 19 kDa pVHL starting from the 2nd methionine at residue 54 was a major functional product.[25–27] This conserved region seems to be critical in the functioning of VHL as a tumor suppressor. Interestingly, non-missense mutations, including micro-deletion, insertion, nonsense, and splice-site mutations, are scattered over the entire conserved region. The missense mutations appear to be localized in 3 regions, codons 78–98, 111–131, and 157–178, that may correspond to functional domains including elongin, Sp1, and PKC binding sites (Fig. 1, Table I).[18, 27, 28] In fact, it has been shown that some of the naturally occurring missense mutations within

the elongin binding domain, including Leu158Pro, Cys162Phe, Cys162Phe and Arg167Gln, abrogated the ability of the VHL protein to bind elongin B/C.[29, 30] Interestingly, there is another missense-cluster region in exon 1 (codons 78–98), strongly suggesting the presence of some important functional domain(s).

A 3-bp deletion mutation between nucleotide 437 and 442 leading to 1 amino acid deletion (delPhe76) was detected in two type 1 families (families 2 and 80). We have previously demonstrated that the VHL patient in family 2 had a *de novo* mutation.[31] Germ-line mutations in families 2 and 80 therefore appear to have occurred independently. DNA sequences around codon 76 contained 2 (TCT) tandem repeats, suggesting that this mutation might be brought about by misalignment-mediated errors in DNA replication, although 4 potential deletion patterns were proposed (Fig. 2). The identical mutation has been reported in at least 10 Western type 1 families,[8, 32] but, so far, has not been found in sporadic tumors as a somatic mutation.[2–7, 22] These observations suggested that this deletion mutation may be one of the hotspots in the type 1 VHL family, while germ cell and somatic cells have distinct mutational mechanisms of the *VHL* gene.

We characterized a total of 37 single base substitutions in germ-line VHL mutations. The pattern of DNA change was similar to that in Western VHL, despite the many differences in diet, life-style and geographical location (Table V). The mutational processes usually involve endogenous processes, exogenous (environmental) mutagens, or both. Environmental mutagens are known to produce characteristic mutation patterns.[33, 34] If exogenous mutagens were responsible for germ-line mutations, different populations should have variable patterns of mutations depending on the specific environmental mutagens involved. Conversely, endogenous processes, which include deamination, depurination, and errors in replication or repair of the gene, should be associated with an invariant pattern of mutations. Similarities in the mutations and the patterns of DNA change between Japanese and Western VHL may reflect endogenous mutation processes rather than environmental mutagens.[33, 34] In addition, transitions at the dinucleotide CpG were a hotspot mutation both in Japanese and Western VHL, although CpG mutations have a higher incidence in Western patients. CpG dinucleotides are well known as mutational targets in human genetic disease.[35] CpG dinucleotides are often methylated. Cytosine and 5-methylcytosine residues can spontaneously deaminate to uracil and thymine, respectively, which, if not repaired, will result in G:C to A:T transitions.[33] The high incidence of transitional mutations at CpG dinucleotides in germ-line VHL appears to be consistent with mutagenesis via endogenous deamination mechanisms. The *VHL* gene may be methylated in germ cells.

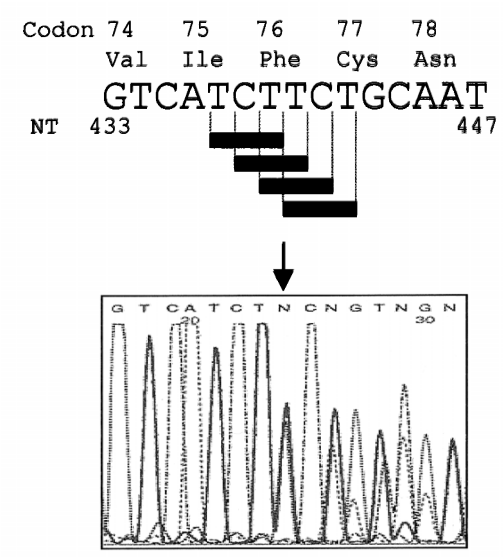

Fig. 2. Upper panel: the sequence of *VHL* gene around codon 76 showing the 3-bp deletion mutation in families 2 and 80. Boxes indicate 4 potential nucleotide deletions. Lower panel: an electropherogram of DNA from the patient in family 80 showing

We also identified several interesting characteristics in Japanese VHL. In the type 1 families, the incidences of nonsense and Southern mutations were significantly lower compared with Western VHL (Fisher's exact test: $P=0.0031$, and 0.0086, respectively) (Table III). In addition, 18 intragenic mutations were newly found in our type 1 patients. In the type 2 VHL families, we identified 2 novel mutations (Arg131Ser and 776ins20-bp). Furthermore, we found that 4 mutations (Arg113Stop, Gln132Stop, Leu158Val, and Cys162Tyr) previously characterized as VHL type 1 mutations occurred in the type 2 Japanese family (Table I).[8] These mutational characteristics may reflect ethnic or environmental factors in Japanese-Asian populations.

We examined genotype-phenotype correlation in our VHL family panel and found that renal cell carcinomas occurred predominantly in non-missense mutations predicted to lead to gross alteration or complete loss of the pVHL. Somatic VHL mutation is frequent in sporadic clear cell RCCs. Moreover, the majority (70–75%) are non-missense mutations including micro-deletion, insertion, and nonsense mutations.[3, 4, 22] These similarities between germ-line and somatic mutations suggested that gross disruption or complete loss of the pVHL might be one of the critical genetic events in the development of clear-cell RCC. The *VHL* gene appears to act as a classical tumor suppressor with "gatekeeper" function in the pathogenesis of clear-cell RCCs.[33] On the other hand, with regard to the tumorigenic mechanisms of pheochromocy-

toma, missense mutations within the elongin binding domain are indeed predominant in the type 2 VHL with pheochromocytoma. However, germ-line missense mutations in the elongin interacting sites do not always lead to pheochromocytomas. In addition, we and others have found that some of the non-missense mutations, which lead to gross alteration of the pVHL, are also involved in the type 2 families.[8, 32] Moreover, somatic VHL mutation is infrequent in sporadic pheochromocytomas, in clear contrast to sporadic RCCs.[36] These complicated relationships between VHL mutation and the occurrence of pheochromocytomas suggested that the *VHL* gene might act as a modifying gene rather than a "gatekeeper" in the tumorigenesis of pheochromocytoma.

We performed SSCP, sequencing of the entire coding exons and intron junctions, and Southern analysis for detecting VHL mutations, and found germ-line mutations in 55/77 (73%) Japanese families. In these VHL families with mutations, accurate presymptomatic genetic testing was feasible. However, we have not yet identified germ-line abnormalities in nearly 30% of the families. Other molecular genetic approaches to improve the mutation detection rate will be necessary in such cases. Recently, Stolle *et al*.[32] demonstrated that germ-line large deletions losing the entire *VHL* gene can be detected by quantitative Southern blotting combined with fluorescence *in situ* hybridization (FISH) analyses. It will be necessary to apply these methods to our apparently mutation-negative cases.

Our data in Japanese-Asian VHL patients, added to those from other groups, confirm the diversity of VHL germ-line mutations. Further detailed analyses of germ-line and somatic mutations, as well as functional studies of the *VHL* gene together with selected mutations, will be critical for a better understanding of VHL disease and VHL-inactivated tumors.

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