

The N131S Mutation in the von Hippel-Lindau Gene in a Japanese Family with Pheochromocytoma and Hemangioblastomas

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Abstract. Von Hippel-Lindau (VHL) disease (VHLD) is a hereditary autosomal dominant syndrome that causes various benign and malignant tumors. VHLD is caused by mutations in the VHL tumor suppressor gene. Here, we report a mutation in the *VHL* gene in a Japanese family with VHLD type 2A, characterized by pheochromocytoma (PHE), and hemangioblastomas (HAB) in both the retina and thoracic spinal cord but without renal cell carcinoma (RCC). We identified a heterozygous A to G point mutation at the second base of codon 131 of the VHL protein (pVHL). This mutation was predicted to convert codon 131 from asparagine to serine (N131S). Although most mutations in VHLD type 2A have been detected in the α domain of pVHL, the present mutated amino acid was located at the region encoding the β domain of pVHL. Previous patients with the N131K or N131T mutation in pVHL developed VHLD type 2B with RCC or VHLD type 1 without PHE, respectively. We also identified somatic loss of heterozygosity (LOH) at chromosome 3p25–26 in the adrenal tumor of the patient. The results of our study suggest that not only the location of mutation but also the altered amino acid may be critical for determining the clinical phenotype of VHLD. LOH was associated with the development of PHE in a patient with the N131S mutation in pVHL.

Key words: von Hippel-Lindau disease, Pheochromocytoma, Hemangioblastoma, DNA diagnosis, Renal cell carcinoma, Loss of heterozygosity

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VON Hippel-Lindau (VHL) disease (VHLD) is an autosomal dominant hereditary disease that is caused by mutations in the VHL tumor suppressor gene on chromosome 3p25–26 [1]. VHLD is characterized by pheochromocytoma (PHE) and/or highly vascularized

tumors such as renal cell carcinoma (RCC) and hemangioblastoma (HAB) in the cerebellum, retina, and spinal cord as well as islet cell tumors in the pancreas [2]. Families with VHLD have been reported to manifest a low (type 1) and high (type 2) risk of PHE. Type 2 is further classified into three types: low (type 2A), high (type 2B) with risk of RCC, and PHE alone without RCC or HAB (type 2C) [3].

The *VHL*-encoded protein (pVHL) comprises 213 amino acids and possesses two functional subdomains, namely, α and β [4]. The α domain extends from codon 158 to 189 and binds Elongin C, which in turn binds Elongin B, Cullin-2, and Rbx1 to form a ubiquitin

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ligase complex. On the other hand, the β domain extends from codon 63 to 157 and binds target molecules such as the α subunits of hypoxia-inducible factor (HIF) that causes proteasome-mediated degradation of hypoxia-inducible molecules in the presence of oxygen [5]. pVHL is also reported to play a role in extracellular matrix formation [6] and is necessary for cell cycle exit [7]. Most VHL type 1 cases are the result of a gross alteration in or the complete deletion of pVHL [2]; this suggests that the simple loss of pVHL function is unlikely to lead to the development of PHE. On the other hand, most mutations that affect VHL type 2 are missense point mutations located at the region encoding the α domain of pVHL [2]. The L188V mutation, also located at the region encoding the α domain of pVHL, was reported to cause VHL type 2C [3]. These results suggest that the mechanism by which PHE develops may be different from that by which HAB or RCC develops. Moreover, the mechanism responsible for the development of VHL type 2A or 2B that also causes both PHE and HAB and/or RCC has not been well described to date.

In this study, we report and discuss the clinical characteristics of a Japanese family with VHL type 2A caused by a mutation located at the region encoding the β domain of pVHL in the *VHL* gene. Although the mutation *per se* was not novel [8], we identified a loss of heterozygosity (LOH) in the affected adrenal gland of one of the patients in this family. This family case study may be helpful to understand the mechanism by which both HAB and PHE develop in VHL.

Materials and Methods

Case report

A 25-year-old male was admitted to our hospital. He was diagnosed with PHE caused by a right adrenal tumor at the age of 13 years and underwent right adrenalectomy. HAB in the thoracic spinal cord and left adrenal tumor were identified at the age of 18 years. Left retinal HAB was diagnosed at the age of 22 years, leading to a clinical diagnosis of VHL type 2A. The patient's father and younger brother were also diagnosed with VHL type 2A. The pedigree of his family is shown in Fig. 1. His father was diagnosed with PHE caused by left adrenal tumor, HAB in the central nervous system (CNS), retinal HAB, and pancreatic

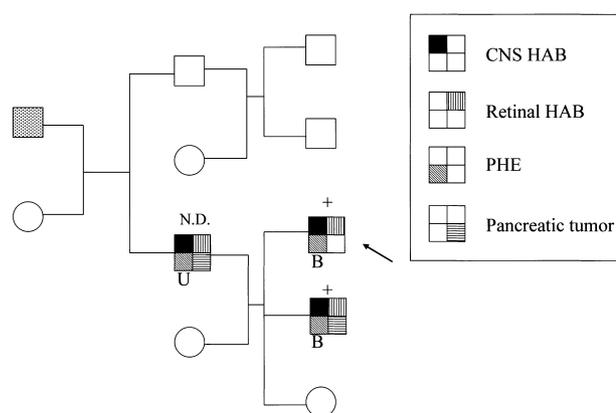


Fig. 1. Pedigree and clinical phenotypes of family members. Arrow indicates the patient. Black and white squares indicate the positive and negative phenotypes, respectively, for CNS HAB, retinal HAB, PHE, or pancreatic tumors. Left upper square: CNS HAB, right upper square: retinal HAB, left lower square: PHE, right lower square: pancreatic tumors. U and B indicate unilateral and bilateral adrenal tumors, respectively. + indicates the N131S germline mutation in pVHL. Gray square represents an unknown clinical phenotype.

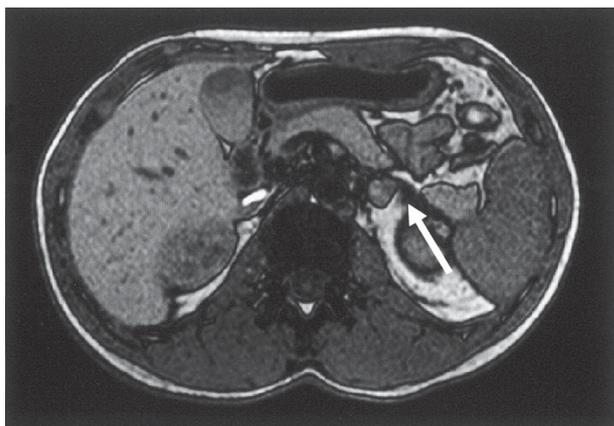
Table 1. Laboratory data of the patient

p-ADR	<0.01 ng/ml	(0–0.17)
p-NAD	0.45 ng/ml	(0.15–0.57)
p-DOA	<0.02 ng/ml	(0–0.03)
u-VMA	5.2 mg/day	(1.4–4.9)
u-ADR	7.8 μ g/day	(1–23)
u-NAD	287 μ g/day	(29–120)
u-DOA	940 μ g/day	(100–1000)
u-meta	0.05 mg/day	(0.05–0.2)
u-nmeta	0.54 mg/day	(0.1–0.28)

Normal range is indicated in parentheses. ADR: adrenalin; NAD: noradrenaline; DOA: dopamine; VMA: vanillylmandelic acid; meta: metanephrine; nmeta: normetanephrine

tumor, whereas his younger brother had PHE caused by bilateral adrenal tumors, pancreatic tumor, and HAB in the CNS and retina. Information regarding the medical history of his grandfather was not available. Since the ^{131}I -meta-iodobenzylguanidine (MIBG) scintigram performed at this time revealed poor uptake of radioactivity into the patient's left adrenal tumor and since his blood pressure was normal, left adrenalectomy was not performed; the size of the left adrenal tumor was followed up by magnetic resonance imaging (MRI) once a year. MRI examination at the age of 24 years revealed an enlargement of the HAB in the spinal cord of the patient, and it was thought to be an indication of surgical operation for HAB. In addition, the ^{131}I -MIBG scinti-

T1-weighted image



T2-weighted image



Fig. 2. Abdominal MRI. Left adrenal tumor (arrow) showed a low intensity in the T1-weighted image and a high intensity in the T2-weighted image.

T1-weighted image



T2-weighted image

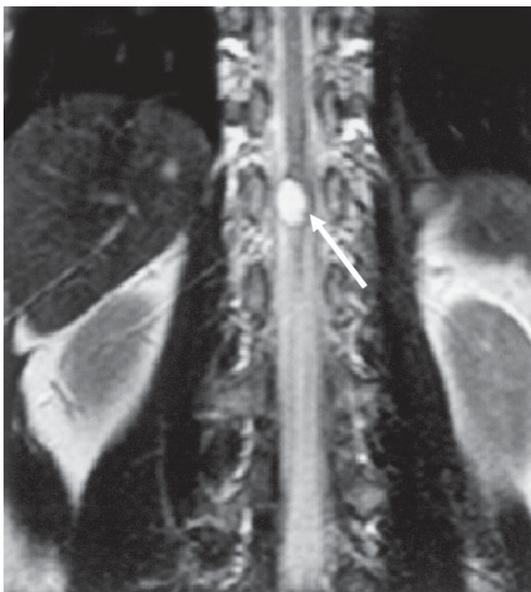


Fig. 3. HAB with cystic mass in the spinal cord at the Th12 level. Tumor showed a low intensity in the T1-weighted image and a high intensity in the T2-weighted image.

gram revealed an increased uptake of radioactivity, and the patient's urinary vanillylmandelic acid (VMA) level was significantly elevated. It appeared that PHE had become functional and left adrenalectomy was considered to be necessary prior to the surgical operation of the HAB. The patient was then admitted to our hospital. His blood pressure and pulse rate were normal. His plasma and urine catecholamine data are shown in Table 1. Abdominal MRI revealed a left adrenal tumor

with a low intensity in the T1-weighted image and a high intensity in the T2-weighted image. In addition, a cystic tumor was identified in the spinal cord at the Th12 level (Fig. 2). As the ^{131}I -MIBG scintigram revealed an increased uptake of radioactivity into the left adrenal gland (Fig. 3), we diagnosed the left adrenal tumor as PHE, and left adrenalectomy was performed. The diagnosis of PHE was confirmed histologically.

Gene analysis

Informed consent was obtained from the patient for analysis of the *VHL* gene, and the study was approved by the ethical committee of the Kobe University Graduate School of Medicine (approval number: 274).

Sequence analysis of the *VHL* gene was performed as described previously [9]. Briefly, peripheral blood samples from the patient and normal control were collected, and genomic DNA was extracted using the Wizard Genomic DNA purification kit (Promega Corp., Madison, WI, USA) according to the manufacturer's instructions. The left adrenal tumor was surgically resected with the normal gland of the patient and fixed with paraffin. Genomic DNA was extracted from the tumor and normal adrenal gland by using Takara DEXPAT[®] (Takara, Tokyo, Japan) according to the manufacturer's instructions. Exons 1, 2, and 3 of the *VHL* gene were then amplified by PCR using primer pairs described previously [10]. After purifying the PCR products by using the QIA quick PCR purification kit (Qiagen Corp., Valencia, CA, USA), direct sequencing was performed using the ABI Prism 310 Genetic Analyzer (model 310; Perkin-Elmer, Applied Biosystems, Foster City, CA, USA).

PCR-based restriction fragment length polymorphism analysis

To confirm the presence of a mutation, PCR-based restriction fragment length polymorphism (RFLP)

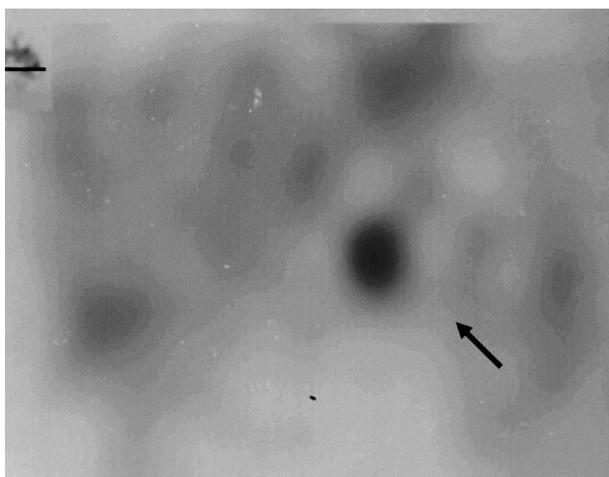


Fig. 4. ¹³¹I-MIBG scintigram showed an increased uptake of radioactivity into the left adrenal tumor.

analysis of exon 2 of the *VHL* gene was performed. After amplifying exon 2 in the *VHL* gene from the patient and normal control, the PCR products were digested with the restriction enzyme *Hpa*I (Takara) according to the manufacturer's instructions. The digested fragments were separated on a 3% agarose gel (Nippon Gene Corp., Tokyo, Japan) and visualized by ethidium bromide staining.

LOH analysis

Microsatellite LOH analysis by PCR was performed using primer pairs D3S1038 as described previously [11]. The PCR products were separated on a 3% NuSieve 3:1 agarose gel (BioWhittaker Molecular Applications, Rockland, ME, USA) and visualized by ethidium bromide staining.

Results

Direct sequencing

As shown in Fig. 5a, by using blood samples, a heterozygous A to G germline mutation was detected at the second base of codon 131 in the *VHL* gene of the patient, whereas only an A signal was observed at this codon in the *VHL* gene of the normal control. This mutation was predicted to convert codon 131 from asparagine to serine (N131S).

On the other hand, only a mutated G signal was detected at the second base of codon 131 in the *VHL* gene from the resected PHE of the patient, whereas a heterozygous A to G alteration was observed at this codon in the *VHL* gene from the normal adrenal gland of the patient (Fig. 6a).

PCR-RFLP

The wild-type *VHL* gene contains a recognition site for *Hpa*I; however, the A to G mutation at the second base of codon 131 disrupts this site. By using *Hpa*I treatment, the 241-bp PCR product from the blood sample of the normal control was completely cut into two bands of sizes 171 bp and 70 bp, while that obtained from the patient's blood sample was partially digested to produce three bands of sizes 241 bp, 171 bp, and 70 bp (Fig. 5b). This result confirmed the presence of a heterozygous A to G germline mutation

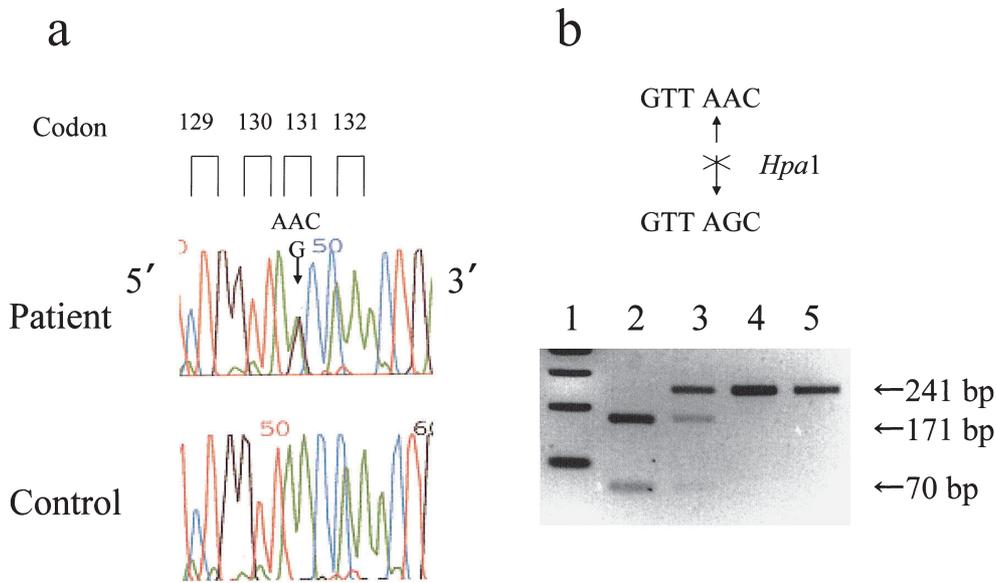


Fig. 5. (a) Direct sequence analysis of the *VHL* gene by using the peripheral blood DNA of the patient as a template. A heterozygous A to G mutation (arrow) was identified at the second base of codon 131. (b) PCR-RFLP analysis by *HpaI*. A 241-bp PCR product from the blood sample of the normal control was completely digested into two bands of sizes 171 bp and 70 bp (lane 2), while that from the blood sample of the patient was partially digested to produce three bands of sizes 241 bp, 171 bp, and 70 bp (lane 3). Lane 1: molecular weight marker, lane 2: PCR product from the normal control (digested with *HpaI*), lane 3: PCR product from the patient (digested with *HpaI*), lane 4: PCR product from the normal control (undigested), lane 5: PCR product from the patient (undigested).

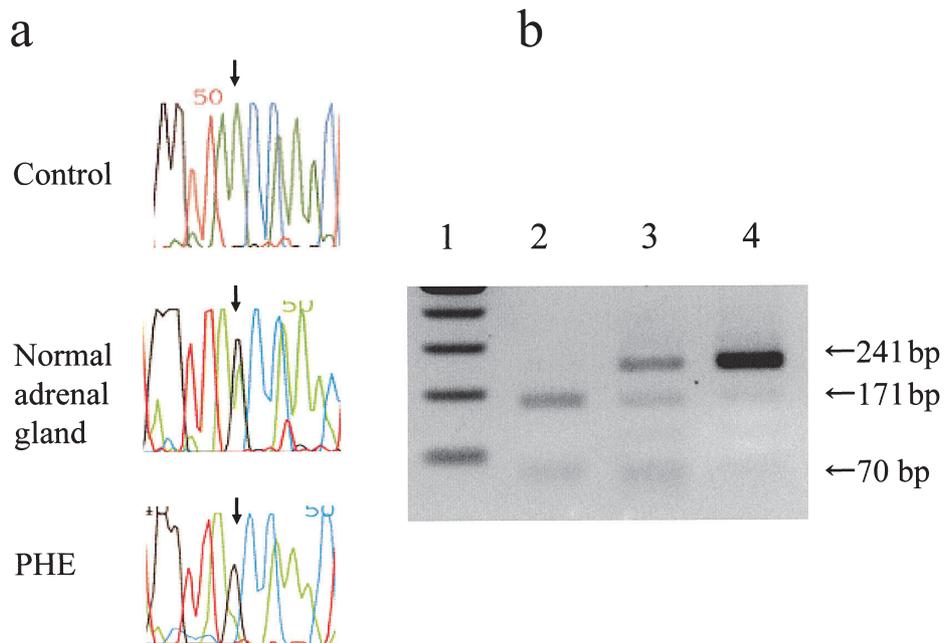


Fig. 6. (a) Direct sequence analysis of the *VHL* gene from the blood sample of the normal control (upper panel), the normal adrenal gland of the patient (middle panel), and the adrenal tumor of the patient (lower panel). A heterozygous A to G mutation was detected in the *VHL* gene from the patient's blood sample, whereas only mutated G signal was detected in the *VHL* gene from the adrenal tumor. (b) PCR-RFLP analysis by *HpaI*. PCR product from the patient's adrenal gland was partially digested, while that from his adrenal tumor was not digested. Lane 1: molecular weight marker, lane 2: PCR product from the blood sample of the normal control, lane 3: PCR product from the resected normal adrenal gland of the patient, lane 4: PCR product from the resected adrenal tumor of the patient.

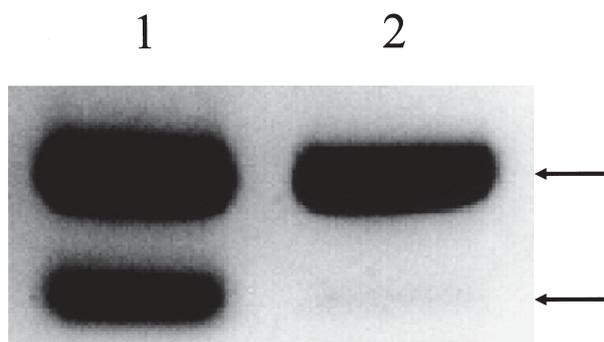


Fig. 7. Microsatellite analysis of the 3p25–26 locus. PCR product from the patient's peripheral blood sample showed two bands, whereas that from his adrenal tumor showed only one band. Lane 1: PCR product from the blood sample of the patient, lane 2: PCR product from the adrenal tumor of the patient.

in the *VHL* gene of the patient.

Similarly, PCR-RFLP confirmed the presence of a mutated allele(s) only in the *VHL* gene from the PHE of the patient (Fig. 6b). The faint bands visible at 171 and 70 bp in lane 4 suggested contamination of the blood in the PHE.

LOH analysis

As shown in Fig. 7, two bands of PCR products were amplified at the D3S1038 locus when the DNA from the blood sample of the patient was used as a template, whereas only one band was amplified when the DNA from the adrenal tumor of the patient was used as a template. Considered together with the result presented in Fig. 6, LOH at chromosome 3p25–26 was likely to occur in the DNA in the PHE of the patient.

Discussion

In this study, we investigated the clinical phenotype of a Japanese family with VHL type 2A with N131S mutation in the *VHL* gene. Although the N131S mutation was not novel and has been reported previously [8], we identified somatic LOH at the 3p25–26 locus in the affected adrenal gland as well as a germline mutation in one of the patients in this family.

Most mutations that affect VHL type 2 are missense mutations located at the region encoding the α domain of pVHL [2]; however, codon 131 is located at the region encoding the β domain. Thus, the missense

mutation observed in the β domain in our patient is relatively rare. Although several mutations have been identified in VHL type 2A to date, 30 cases of VHL type 2A without missense mutations in the α domain have been reported; these include 20 cases of missense mutations in the β domain; 1 large deletion; 1 microdeletion; 5 rearrangements; 2 insertions; and 1 frameshift mutation [12–18].

Although the genotype-phenotype correlation and pathophysiology of VHL have been discussed and studied by many groups, the precise mechanism by which PHE develops remains to be clarified.

Several hypotheses have been proposed with regard to the pathogenesis of PHE. The inhibition of HIF1 α activation by the *VHL* gene mutation is not always consistent with the pathogenesis of PHE since the mutation causing VHL type 2C failed to inhibit HIF activation [19]. Another hypothesis is that the mutation that results in a single amino acid change in pVHL behaves as a gain-of-function, leading to its binding with another protein in the functional pathway [20]; however, no clear evidence is available to support this hypothesis. Recently, an alternative hypothesis regarding the development of PHE has been proposed. It is suggested that the mutation in the *VHL* gene decreases the activity of a 2-oxoglutarate-dependent oxygenase, namely, SM-20/EgIN3/PHD3, resulting in reduced apoptosis of the neural crest cells during development [21, 22]. Okuda *et al.* [22] reported that the mutated pVHL in VHL type 2C failed to ubiquitinate the atypical protein kinase C group, resulting in an increase in c-jun that inhibits apoptosis; this led to the development of PHE. These reports suggest that PHE in VHL requires a reduction in the ability of pVHL to facilitate apoptosis rather than an impairment of HIF degradation. The same group previously reported that the β domain of pVHL interacted with the regulatory domain of atypical protein kinase C (PKC) isoforms and was required for apoptosis regulation [23]. In our study, the detection of the N131S mutation at the region encoding the β domain and not the region encoding the α domain of pVHL may support the latter hypothesis; this indicates that PHE in VHL with the N131S mutation may be caused by the reduced apoptosis of the neural crest cells rather than reduced HIF degradation. However, although this hypothesis holds true for the N131S mutation, it does not for most of the other missense mutations located at the region encoding the β domain because most of these mutations

caused VHL type 1 without PHE. On the other hand, it is reported that gene dosage effect disturbs cell proliferation, differentiation, and apoptosis without requiring a second hit. Tumorigenesis of PHE could be induced by a 50% reduction in the normal pVHL level [20]. In our study, however, in addition to the N131S germline mutation, we identified a second hit by LOH at 3p in the PHE. A previous report by Bender *et al.* showed that the LOH at 3p was detected in 91% PHE with VHL; this value was relatively high when compared with 24% incidence observed with the LOH at 3p in sporadic PHE [24]. This is in agreement with the result of our present study.

In previous studies, the missense mutation detected in codon 131 showed various clinical phenotypes. Stolle *et al.* [16] reported that individuals with an N131T mutation in pVHL showed no signs of PHE, whereas our patient who had an N131S mutation developed PHE. Furthermore, Gallou *et al.* [25] reported that an N131K mutation caused RCC; however, no RCC was detected in our patient who had an N131S mutation in pVHL. In previous report by Yoshida *et al.*, it was revealed that an N131S mutation in pVHL leads to the development of PHE and HAB [8]; this is in agreement with the clinical phenotypes observed in our patient. In that previous study, a family with the N131S mutation was reported that comprised 2 patients, both of whom demonstrated retinal HAB and PHE, and one of whom also had CNS HAB and a pancreatic tumor [8]. On the other hand, as shown in Fig. 1, all 3 patients in our study had CNS HAB, retinal HAB, and PHE, and 2 of them also had pancreatic tumors, hence these clinical phenotypes were similar to those reported in the previous study [8]. Thus, the clinical phenotypes of VHL may depend not only on the mutation site but also on the altered amino acid. Muta-

tions occurring at the same site with different amino acids and demonstrating different clinical phenotypes of VHL were also reported in codons 65, 93, 161, 162, and 167. For example, the S65L mutation, but not the S65W mutation, was associated with PHE [4, 18, 26]. Similarly, the R161Q mutation, but not the R161T mutation, was associated with PHE [9, 26]. Furthermore, the R161G mutation was associated with RCC as well as HAB and PHE [13]. Thus, an alteration in the amino acid may contribute to the clinical phenotype of VHL, including PHE, HAB, and RCC. Since the relationship between the mutated amino acid and clinical phenotypes has not been well investigated thus far, our hypothesis that an altered amino acid may be crucial for determining the clinical phenotype of VHL is a novel one. Since the missense mutations in the region encoding the β domain of pVHL in VHL type 2 are relatively rare, further clinical observation or functional studies are required to clarify the mechanism responsible for the clinical phenotype of VHL.

In conclusion, we reported the clinical phenotype of an N131S mutation located at the region encoding the β domain of pVHL in a Japanese family with VHL type 2A. This relatively rare mutation allows us to hypothesize that not only the location of mutation but also the altered amino acid may be critical for determining the clinical phenotype of VHL.

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