

p.N78S and p.R161Q germline mutations of the *VHL* gene are present in von Hippel-Lindau syndrome in two pedigrees

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Abstract. Von Hippel-Lindau (VHL) disease is an autosomal dominant familial cancer syndrome. VHL is characterized by the development of renal cell carcinoma (RCC), hemangioblastomas of the central nervous system or retina and pheochromocytoma (PCC). RCC and PCC are known to be caused by germline mutations of six and ten genes, respectively. In the present study, 30 individuals from two unrelated pedigrees with type 2A and 2C VHL syndrome were investigated. The patients were clinically examined and treated by radical nephrectomy [or nephron-sparing surgery (NSS)] and cortical-sparing adrenalectomy (CSA), and all members of the two families underwent genetic screening. Two members from the first family were diagnosed with PCC and RCC, and three individuals from the second family who had only hypertension were diagnosed with PCC. Heterozygous variants of the *VHL* gene, c.A233G (p.N78S) within exon 1 and c.G482A (p.R161Q) within exon 3, were verified, respectively. Surgery was performed on all the patients, with the exception of an asymptomatic 5-year-old p.N78S male in family 1, in addition to genetic testing and genetic counseling. Further patient

follow-up was warranted with regard to blood pressure and health, although normal blood pressure and no local recurrence and distant metastasis of VHL were observed previously. The present study suggests that molecular genetic testing may aid the diagnosis and clinical management of VHL syndrome.

Introduction

Von Hippel-Lindau (VHL) disease (OMIM 193300) is an autosomal dominant familial cancer syndrome that increases susceptibility to several benign and malignant tumors, including central nervous system (CNS) hemangioblastomas, renal cysts and renal cell carcinoma (RCC), as well as pheochromocytoma (PCC). Additional tumors, such as pancreatic and endolymphatic sac tumors and epididymal and broad ligament cystadenomas, have also been previously described (1-6). VHL syndrome accounts for ~1/3 of patients with CNS hemangioblastoma, >50% of those with retinal angioma, 1% of those with RCC, 50% of those with apparently isolated familial PCC and 11% of those with an apparently sporadic PCC (7-9). VHL is present at a frequency of 1 in 36,000 live births in Eastern England (2,5). Similar to other hereditary syndromes, VHL has its own clinical classification based on genotype-phenotype associations. Based on clinical symptoms, patients with low-risk PCC (<5%) are classified as VHL type 1 and those with high-risk PCC (>60%) as VHL type 2. VHL type 2, according to 7-20% of VHL kindreds, is further subdivided into three hypotypes, 2A (patients with a low risk for RCC), 2B [patients with an increased risk (~70%) for RCC] and 2C (affected members with isolated PCC) (1,2,10).

VHL, the gene responsible for VHL disease, was identified in 1993 (11). It is a pleiotropic tumor suppressor gene located on chromosome 3 (3p25.3) that is widely expressed in fetal and adult tissues (11,12). Several *VHL* germline mutations have been identified in individuals with VHL disease, as well as somatic mutations in sporadic tumors (5,13-16). VHL type 1 families frequently harbor larger deletions and nonsense mutations in addition to missense variants in the *VHL* gene, and

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patients with type 2 disease carry missense mutations almost exclusively (5,10). Up to 96% of patients with PCC have missense mutations (10).

The first *VHL* case with a germline mutation in China was reported by Huang *et al* (17) in 2004. In the present study, two frequent *VHL* germline mutations, c.A233G (p.N78S) and c.G482A (p.R161Q), are described in two families with type 2A and 2C *VHL* syndrome. Patients underwent surgery and were subsequently followed up.

Materials and methods

Patients. Two *VHL* syndrome pedigrees were investigated in this study; family 1, a three-generation pedigree including 6 individuals (Fig. 1A), and family 2, a four-generation pedigree containing 24 individuals (Fig. 1B). The present study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committees of the 117th PLA Hospital and Zhejiang University (Hangzhou, China). Written informed consent was provided by all the subjects in this study.

In family 1, the proband (F1-II-1), a 31-year-old male patient, presented with headache, paroxysmal hypertension (150-178/100-120 mmHg) and dizzy spells in 2009 with a 4-year history. Ultrasound (US) and magnetic resonance imaging (MRI) scans confirmed single masses, multiple tumors and multiple cysts in the right adrenal glands, bilateral kidney and pancreas, respectively (Fig. 2A and B). Following treatment with α -blockers, the patient underwent right adrenal tumorectomy and right nephron-sparing surgery (NSS). Right PCC and multiple right RCCs (Fuhrman Grade 2) were confirmed by histopathological examination. In 2012, the patient was diagnosed with bilateral epididymal head multiple nodules. Bilateral epididymal tubercle resection was performed and epididymal papillary adenoma was confirmed by histopathological examination. Additionally, the patient's younger brother (F1-II-3, 30 years old) presented with multiple right renal masses, epididymal nodules and multiple pancreatic cysts (Fig. 2C and D). Radical right nephrectomy was performed and histopathological analysis revealed right RCC with hemorrhagic cyst (Fuhrman Grade 2; Table I).

In family 2, the proband (F2-II-11), a 45-year-old female patient, presented with paroxysmal hypertension (180-210/100-120 mmHg), headache, palpitations and hyperhidrosis in 1985 at the age of 18. US scans showed an isolated mass in the right adrenal gland. Following treatment with α -blockers and phenoxybenzamine or terazosin hydrochloride, the patient underwent right adrenal tumorectomy, and PCC was confirmed by histopathological examination. In 2002, the son of the proband (F2-III-9, 9 years old) also presented with paroxysmal hypertension (140-160/100-110 mmHg) and headache. Biochemical examination indicated increased serum catecholamines, and an isolated mass in the right adrenal gland was confirmed by US and CT scans. Following treatment with α -blockers, right adrenal tumorectomy was performed. Histopathological analysis revealed a right adrenal PCC. In 2010, when the patient was 17 years old, laparoscopic adrenal tumorectomy with carbon dioxide pneumoperitoneum was performed based on the evidence of paroxysmal hypertension, headache, elevated serum catecholamines and

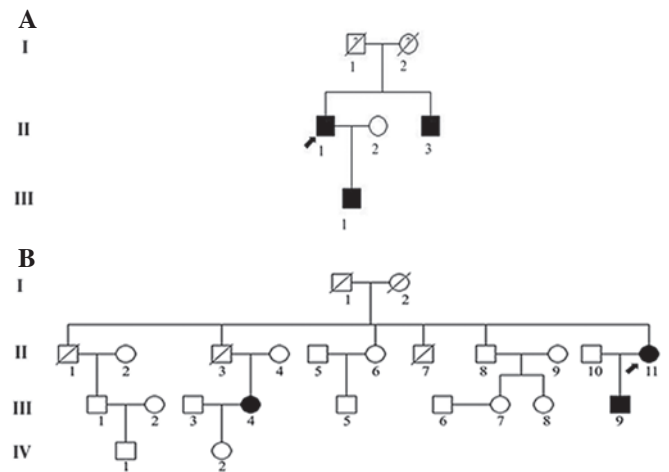


Figure 1. (A) Pedigree 1 with *VHL* type 2A; (B) Pedigree 2 with *VHL* type 2C. Arrows indicate probands. *VHL*, von Hippel-Lindau syndrome.

a mass in the left adrenal gland, but without right adrenal gland abnormalities. PCC was confirmed by histopathological examination. Another patient, the niece of the proband (F2-III-4, 33 years old), presented with headache, sustained hypertension, dizzy spells and excess serum catecholamines. Bilateral masses in the adrenal glands were confirmed by US and CT scans when the patient was 28 years old in 2007. High-dosage $\alpha 1$ blockade with doxazosin mesylate and hydrocortisone cortisol were administered prior to operation, and partial bilateral laparoscopic adrenalectomy with carbon dioxide pneumoperitoneum was performed. Histopathological examination indicated bilateral PCC. The 38-year-old father of this patient (F2-II-3, the proband's brother) succumbed to hypertensive intracerebral hemorrhage 21 years prior to this study (Table I). Among the members of family 2, three were diagnosed with PCC as the only symptom and no other systematic tumors, including CNS hemangioblastomas, renal cysts or RCC, were identified.

All five patients (F1-II-1, F1-II-3, F2-II-11, F2-III-4 and F2-III-9) were followed up. Biochemical and imaging examination did not reveal any evidence of recurrence of PCC and RCC and blood pressure returned to normal, with the exception of one case (patient F1-II-1) where left RCC remained evident and the patient agreed to undergo scheduled left NSS at a later stage. Patient F2-III-4 required steroid replacement therapy. However, patients F2-III-4 and F2-III-9 with bilateral tumorectomy or partial adrenalectomy did not develop adrenocortical insufficiency or Addisonian crisis during the period of observation.

Mutation analysis. Peripheral blood samples were collected for mutation analysis of the *RET* proto-oncogene and *VHL* tumor suppressor gene. Genomic DNA was extracted from whole-blood samples using the QIAamp blood kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Polymerase chain reaction (PCR) amplification of all exons and the flanking splice junctions of *RET* and *VHL* was performed, followed by direct bidirectional DNA sequencing. One hundred unrelated controls with a similar ethnic background were also included in this study.

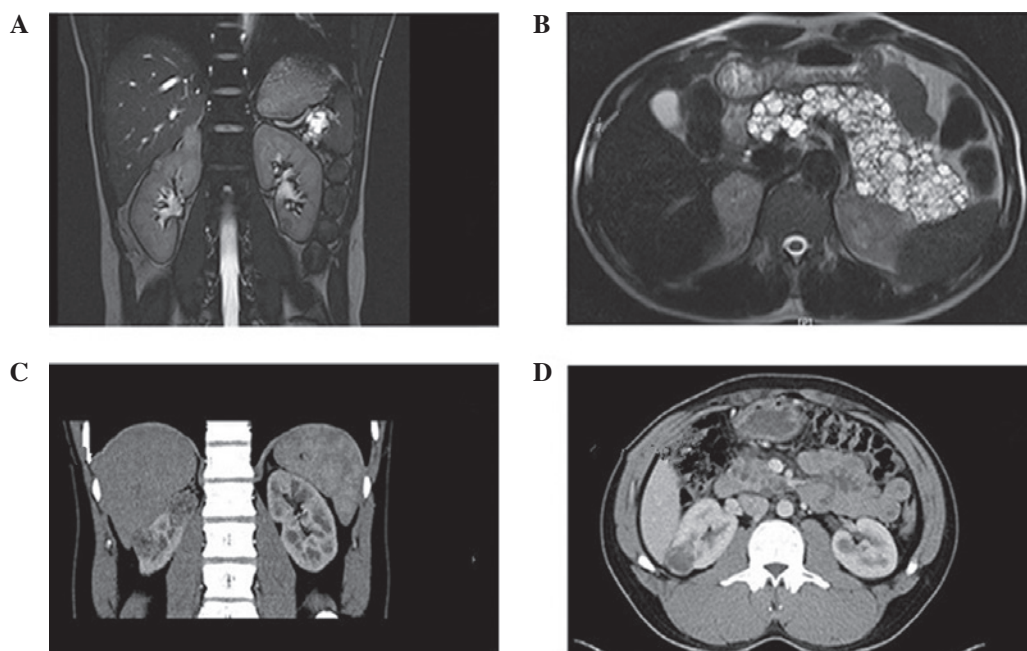


Figure 2. (A and B) A 31-year-old male patient from family 1 (F1-II-1). Abdominal magnetic resonance imaging (MRI) indicated a right adrenal tumor, bilateral multiple renal masses and multiple pancreatic cysts. (C and D) Brother of patient F1-II-1 (F1-II-3, 30 years old). Computed tomography of the abdomen showed multiple solid/cystic lesions in the right kidney and multiple pancreatic cysts. PCC and RCC were confirmed by pathological examination. PCC, pheochromocytoma; RCC, renal cell carcinoma.

Results

In family 1 with type 2A VHL syndrome, we identified a heterozygous variant, c.A233G (p.N78S), within exon 1 of the *VHL* gene in two affected cases and one clinically normal family member (F1-III-1, the proband's 5-year-old son; Fig. 3A). A heterozygous nucleotide substitution within exon 3 of the *VHL* gene, c.G482A (p.R161Q), was identified in the proband (F2-II-11) and two additional cases (F2-III-4 and F2-III-9) of family 2 with VHL type 2C (Fig. 3B). No mutants of the *RET* gene were identified in these cases.

Discussion

VHL disease-related PCC is a tumor of the main sympathetic paraganglion, the adrenal medulla, which is complicated by hypertension. PCC is usually benign, with ~20% malignant cases (18). Molecular genetic studies suggest that ~50% of individuals with apparent familial non-syndromic PCC have *VHL* germline mutations (18). Previous studies have shown that PCC occurs sporadically or as part of hereditary tumor-predisposition syndromes caused by germline mutations affecting one of the ten following susceptibility genes: *VHL*, *RET*, *SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*, *NF1*, *TMEM127* or *MAX* (Table II) (1,2,19-27). The majority of familial cases of PCC and 10-20% of sporadic cases carry germline mutations of these genes (4). RCC represents 90-95% of neoplasms arising from the kidney, which is an important cause of death in VHL disease (7). RCC is rare in children and young adults, and occurs in both a non-familial and familial form. Hereditary RCC accounts for ~4% of RCC, including the following disorders: VHL disease; hereditary leiomyomatosis related to *FH* gene mutation;

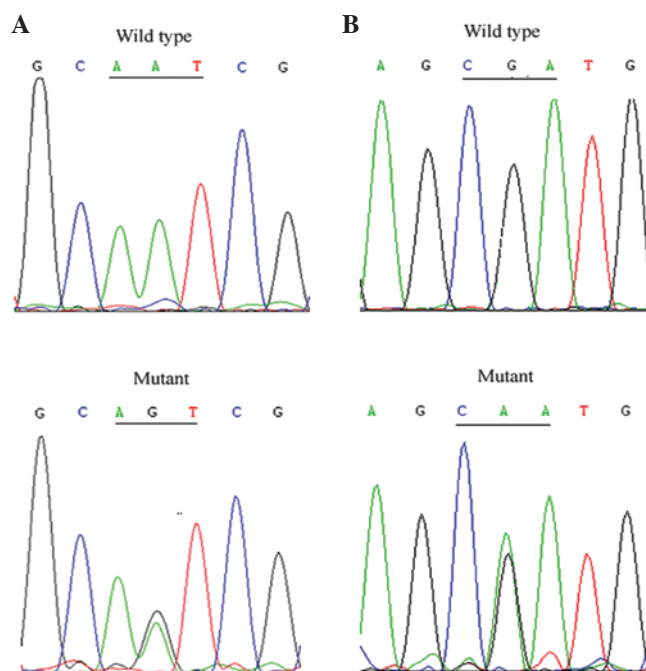


Figure 3. (A) DNA sequencing results showed a heterozygous A-to-G transition at the nucleotide position 233 (p.N78S) of the *VHL* gene in family 1. (B) DNA sequencing results showed a heterozygous G-to-A transition at the nucleotide position 482 (p.R161Q) of the *VHL* gene in family 2.

tuberous sclerosis, which results from the germline mutation of *TSC1* and *TSC2* genes; hereditary papillary renal cell carcinoma (HPRCC), which is associated with *MET* gene; and the gene responsible for Birt Hogg-Dubé syndrome, which has been reported to be the *FLCN* gene (Table II) (28-31). In a familial form, RCC occurs at an early age (with

Table I. Clinical presentations of RCC and PCC with VHL mutations.

Individual	Gender	Age at onset (years)	VHL mutation	Catecholamine serum level (ng/l) (normal: DA, 100; E, 600; NE, 100)	Tumor size (cm)			Operation	Histology
					PCC	RCC	RCC		
F1-II-1	M	31	p.N78S	-	R, 4.1x3.4x3.9	R, 1.0x1.0x1.0; L, 1.0x1.0x0.8	R-Tumorectomy; R-NSS; Scheduled left NSS later	R-PCC, Bi-RCC	
F1-II-3	M	30	p.N78S	-	-	R, 4.0x3.0x2.5	Radical R-nephrectomy	R-RCC	
F1-III-1	M	5	p.N78S	-	-	-	-	-	
F2-II-11	F	18	p.R161Q	-	R, 5.5x4.5x2.8	-	Tumorectomy	R-PCC	
F2-III-9	M	9(R)/17(L)	p.R161Q	Elevated (DA, 88.64; E, 106.23; NE, 827.15)/Elevated (DA, 600.36; E, 297.94; NE, 1093.84)	L, 7.0x5.5x3.4; R, 5.6x3.8x3.0	-	Tumorectomy	Bi-PCC/He	
F2-III-4	F	28	p.R161Q	Elevated (DA, 268.63; E, 1270.3; NE, 1802.25)	L, 6.0x4.7x3.5; R, 3.5x2.8x2.5	-	Bi-partial adrenalectomy	Bi-PCC/Si	

RCC, renal cell carcinoma; PCC, pheochromocytoma; M, male; F, female; DA, dopamine; E, epinephrine; NE, norepinephrine; R, right; L, left; NSS, nephron sparing surgery; Bi-, bilateral; He, heterochronous; Si, simultaneous.

the exception of HPRCC) and usually presents bilaterally with a high incidence (7,31). According to a recent study, 20/255 patients (7.8%) with hereditary leiomyomatosis and RCC were reported to have primary adrenal lesions, including 4 patients with bilateral adrenal lesions and 4 with multiple nodules (28). Therefore, in future studies on PCC and RCC patients, targeted regions (all exons of *VHL*, *RET*, *SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*, *NF1*, *TMEM127*, *MAX*, *FH*, *TSC1*, *TSC2*, *MET* and *FLCN*) or exome sequencing should be analyzed (32).

The *VHL* gene has been conserved throughout evolution and is found in all multicellular organisms. The two *VHL* gene products, pVHL, a 30-kDa full-length protein [p30, 213 amino acids, NM_000551.2 (variant 1 mRNA)], and a 19-kDa protein [p19, 160 amino acids, NM_198156.1 (variant 2 mRNA)], suppress tumor formation in nude mice and regulate hypoxia-inducible factor- α (HIF- α) (33,34). pVHL has two domains, the β domain (N-terminal β -sheet domain), which contains a macromolecular binding site, and an α domain (N-terminal α -helical domain), which interacts with elongin C (35). The region between codon 75-82 is located in the β -binding domain of VHL protein; thus, the p.N78S mutation interferes with HIF binding, which results in the excessive transcription of HIF target genes, such as vascular endothelial growth factor (13). Codon 161 is located in the α domain and interacts with elongin C. The mutation in the α domain affects VHL tumor suppressor activity (36,37). Through errors in chromosomal replication during cell division, additional genes or modifying factors may be involved in the genetic basis of VHL (37).

VHL genotype-phenotype correlations have been previously established (5,17). The p.N78S mutation has been reported to be associated with PCC, RCC and CNS hemangioblastomas (8). The p.R161Q mutation was first reported by Tong *et al* (38) in Chinese VHL patients who had non-syndromic familial PCC without hypertension and is also known to be associated with retinal and CNS hemangioblastoma and RCC (10). For patients included in the present study, *VHL* mutations were consistent with the type 2A and 2C phenotypes. In particular, p.R161Q was associated with PCC with hypertension syndrome, and p.N78S had a more severe clinical phenotype (RCC and bilateral PCC) compared with p.R161Q. However, none of the mutations were associated with CNS hemangioblastomas.

The identification of the *VHL* tumor suppressor gene facilitates the diagnosis of VHL disease, particularly in patients who do not meet the clinical diagnostic criteria. It also has important implications on the estimation of risk for the development of further tumors in patients and of VHL disease in their relatives. In family 1, a young family member (F1-III-1, 5 years old) did not have an evident clinical phenotype but carried the p.N78S mutation. The identification of the *VHL* tumor suppressor gene is beneficial for the early prevention and treatment of VHL syndrome. Additionally, the mean age at diagnosis of PCC and RCC in VHL is ~30 and ~40 years, respectively (39,40). However, according to a clinical report, a 2.75-year-old female patient with PCC was found to carry a p.R161Q germline mutation (40). This is the youngest patient with PCC reported to date. For patients in the present study, the age of onset for patient F2-III-9 was 9 years, while the other two patients were 18 and 28 years

Table II. Summary of susceptibility genes associated with PCC and RCC.

Gene (location)	Protein function	Clinical phenotype	(Ref.)
<i>RET</i> (10q11.2)	Tyrosine kinase receptor that plays a crucial role in regulating cell proliferation, migration, differentiation and survival through embryogenesis.	Multiple endocrine neoplasia type 2 (MEN 2)-autosomal dominantly inherited predisposition to medullary thyroid carcinoma and PCC. MEN 2A is characterized by medullary thyroid carcinoma, PCC and primary hyperparathyroidism. MEN 2B is characterized by medullary thyroid carcinoma, PCC and developmental anomalies (e.g., Marfanoid habitus and mucosal neuromas). PCCs in MEN 2 are characteristically adrenal and benign.	(22)
<i>VHL</i> (3p25)	Short protein with potent tumor suppressive activity.	Von Hippel-Lindau disease, autosomal dominantly inherited predisposition to PCC, retinal and cerebellar hemangioblastomas, clear RCC, non-secretory pancreatic neuroendocrine tumors, endolymphatic tumors and visceral cysts (renal, pancreatic and epididymal). Rarely head and neck paragangliomas. Epididymal cystadenomas.	(1)
<i>SDHA</i> (5p15)	Flavoprotein catalytic subunit of Complex II.	Autosomal dominantly inherited predisposition to PCC, head and neck paragangliomas (though infrequent cause of these tumors) (autosomal recessively inherited juvenile encephalopathy or neonatal cardiomyopathy).	(23)
<i>SDHB</i> (1p36)	Iron-sulfur protein catalytic subunit of Complex II.	Autosomal dominantly inherited predisposition to PCC, head and neck paragangliomas and renal cell carcinoma. High frequency of extra-adrenal PCC and malignancy.	(24)
<i>SDHC</i> (1q21)	One of the two transmembrane subunits of Complex II of the respiratory chain.	Autosomal dominantly inherited predisposition to head and neck paragangliomas, PCC.	(19)
<i>SDHD</i> (11q23)	One of the two transmembrane subunits of Complex II of the respiratory chain.	Autosomal dominantly inherited predisposition to parasympathetic head and neck paragangliomas and PCC. Tumor development generally occurs only in individuals who have inherited the mutation from their father (i.e., parent-of-origin effects on disease phenotype).	(20)
<i>SDHAF2</i> (11q12.2)	Mitochondrial assembly factor for Complex II, interacts directly with SDHA.	Autosomal dominantly inherited predisposition to head and neck paragangliomas and PCC.	(25)
<i>NF1</i> (17q11.2)	Studies on animal models show that homozygous mutant mice for the NF1 gene display a variety of developmental abnormalities leading to midgestation lethality.	Neurofibromatosis type 1 (von Recklinghausen disease) autosomal dominantly inherited predisposition to PCC, peripheral nervous system tumors (cutaneous, nodular and plexiform neurofibromas), intestinal and gastrointestinal stromal cell tumors, malignant gliomas and juvenile chronic leukemias.	(26)
<i>TMEM127</i> (2q11.2)	Transmembrane protein involved in protein trafficking.	Autosomal dominantly inherited predisposition to PCC and head and neck paragangliomas. Relatively late mean age of diagnosis (compared to VHL disease and SDHB mutations).	(27)
<i>MAX</i> (14q23)	Component of the MYC-MAX-MXD1 network and regulates cell proliferation, differentiation and apoptosis.	Autosomal dominant inheritance of bilateral PCC, confirming the association between genetic abnormalities and multiple tumors. An association with malignancy was also found.	(21)
<i>FH</i> (1q42-43)	FH is a Krebs cycle enzyme that converts fumarate to malate.	Hereditary leiomyomatosis and RCC is an autosomal dominant disorder characterized familial with an aggressive form of papillary kidney cancer and the development of cutaneous and uterine leiomyomas.	(28)
<i>TSC1</i> (9q34) and <i>TSC2</i> (16p13.3)	Proteins act as tumor growth suppressors, agents that regulate cell proliferation and differentiation.	Tuberous sclerosis or tuberous sclerosis complex is a rare multi-system genetic disease that causes non-malignant tumors to grow in the brain and on other vital organs such as the kidneys, heart, eyes, lungs and skin.	(29)

Table II. Continued.

Gene (location)	Protein function	Clinical phenotype	(Ref.)
<i>MET</i> (7q31)	MET transmembrane protein is a tyrosine kinase receptor which induces proliferation and differentiation of epithelial and endothelial cells, cell branching and invasion.	Hereditary papillary renal carcinoma is an autosomal dominant renal cancer syndrome in which affected individuals are at risk of developing multifocal bilateral type I papillary renal carcinoma.	(30)
<i>FLCN</i> (17q11.2)	FLCN forms a complex with folliculin-interacting proteins (FNIP1 and FNIP2). These components intern bind to AMP-activated protein kinase.	Birt-Hogg-Dubé syndrome is an autosomal dominant disease, and affected individuals develop cutaneous fibrofolliculomas, pulmonary cysts, spontaneous pneumothoraces and renal tumors.	(31)

PCC, renal cell carcinoma; RCC, pheochromocytoma; SDHA, succinate dehydrogenase, subunit A; SDHB, succinate dehydrogenase, subunit B.

old when they were diagnosed with PCC. Thus, molecular genetic testing and genetic counseling for patients with PCC and RCC should be performed at an early age. Early diagnosis of VHL would be beneficial, since regular surveillance for tumors and early intervention are suggested to result in better prognosis.

Numerous studies have shown that VHL patients treated with NSS have a high incidence of local recurrence of new primary tumors, but a low risk of distant metastasis. Approximately 25% of VHL patients with more advanced RCC (>3 cm) develop metastatic disease (41,42). Radical bilateral nephrectomy should be performed when RCC >3 cm, and renal transplantation is required. Bilateral PCC is usually treated by CSA (adrenal tumorectomy or partial adrenalectomy). Patients may avoid hormone replacement, but there would be the risk of late recurrence. Total adrenalectomy may be considered as an alternative when there is a high incidence of multiple bilateral adrenal masses and large tumors; however, life-long steroid replacement is required. All the patients should be followed up regularly to aid the identification of the processing adrenocortical insufficiency or Addisonian crisis (3).

In the present study, the p.N78S mutation patient (F1-II-1) with multiple bilateral RCC (1.0 and 1.0 cm) and right PCC (4.1 cm) was subjected to right NSS and right CSA. The left RCCs were observed using imaging and exhibited no marked enlargement, while locoregional recurrence, distant metastasis and/or CNS were not found in two cases (F1-II-1 and F1-II-3) 39 and 6 months after the surgery, respectively. Among the three p.R161Q mutation patients, one (F2-III-9) had a left PCC 8 years after presenting with a right PCC at 9 years of age, one (F2-III-4) had simultaneous bilateral PCC and the other (F2-II-11) only presented with a right PCC at 18 years of age. All of them underwent bilateral or unilateral CSA and adrenocortical function was normal without steroid replacement. Therefore, identification of the underlying mutation not only confirms the diagnosis, but also provides useful information for genetic counseling and subsequent management decision making.

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