

## RESEARCH ARTICLE

# VHL Gene Mutation Analysis of a Chinese Family with Non-Syndromic Pheochromocytomas and Patients with Apparently Sporadic Pheochromocytoma

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### Abstract

**Objective:** The Von Hippel-Lindau syndrome (VHL), an inherited neoplastic syndrome predisposing to central nervous system hemangioblastoma (CNS), pheochromocytoma (PCC), renal cell carcinoma (RCC), retinal hemangioma (RA) and renal cysts, is caused by mutations or deletions of the VHL tumor-suppressor gene. To assess VHL genotype-phenotype correlations with function of pVHL a gene mutation analysis of members in a Chinese family with non-syndromic PCCs and individuals with apparently sporadic pheochromocytoma (ASP) was performed. **Materials and Methods:** DNA samples of 20 members from the Chinese family with non-syndromic PCCs and 41 patients with ASP were analyzed by polymerase chain reaction and direct sequencing, confirmed by Taqman probe. **Results:** Three novel mutations (H125P, 623(↑TTTGTtG) and R120T) were identified in the Chinese family and in 3 among 41 ASP patients. The mutations were all located in exon 2 of VHL gene encoding β-domain of pVHL. The tumor type in H125P carriers and R120T carriers was VHL type 2C. And 623(↑TTTGTtG) carriers presented VHL type 2B or type 2C. **Conclusions:** VHL gene abnormalities were identified in the Chinese family with non-syndromic PCCs and patients with APS, resulting in dysfunction of pVHL. H125P and R120T could be associated with VHL type 2C, while 623(↑TTTGTtG) might be linked with VHL type 2B or type 2C. Not only is the genetic analysis helpful for early diagnosis and treatment of patients with VHL, it is also beneficial for research into VHL pathogenesis.

**Keywords:** von Hippel-Lindau syndrome - gene - mutation - pheochromocytomas - familial - sporadic

*Asian Pac J Cancer Prev*, 16 (5), 1977-1980

### Introduction

VHL gene is a tumor suppressor gene mapped to chromosome locus 3p25-26, encoding two different protein VHL (pVHL30 and pVHL19) that regulates hypoxia-inducible genes, the fibronectin matrix assembly, and angiogenesis. pVHL19 with 160 amino acid protein is located in the nucleus, pVHL30 with 213 amino acid is expressed both in the nucleus and cytoplasm. The pVHL19 is similar to the pVHL30 in biochemistry and function, affecting a series of processes including cell-cycle regulation, mRNA stability, hypoxia-inducible gene expression and transcription, etc (Lonsdale et al., 2003).

The pVHL19 contains two functional subdomains: a smaller, helical α-domain, which consists of three helices (H1, H2, and H3), and a larger β-domain (residues 63-154 and residues 193-204), which forms a seven-stranded β sandwich and an α helix. These two subdomains contain different protein binding sites: The α-subunit is connected to the elongin C-binding site and the β-subunit binds to

a region of HIF-α that regulates the expression of VEGF (Binderup et al., 2013). Under the conditions of hypoxic and dysfunctional of pVHL, the expression of HIF-α and VEGF may rise, which may promote tumor incidence and growth. VHL gene mutations is related to the pathogenesis of VHL (Gao et al., 2013)

According to genotype and phenotype, VHL can be divided into two types: Type I not with pheochromocytoma (PCC) and Type II with PC. According to whether patients have renal cysts and renal cell carcinoma (RCC), Type II patients are further divided into three sorts: type 2A with low risk of renal cysts and RCC, type 2B with high risk of renal cysts and RCC and type 2C with isolated PCC. The incidence of VHL patients with PCC is about 10% to 34%. Most of them have typical clinical presentation, but a few are asymptomatic (Jimenez et al., 2006). The main causes of VHL type I are correlated with missense mutations and partial deletions, which cause parts of dysfunction or a complete defect of protein function. On the other hand, the causes of VHL type II are usually

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related to missense mutations affecting pVHL to connect to the binding sites. It was suggested by a report that the estimate of hereditary disease in apparently sporadic pheochromocytoma (SPA) was as high as 24% (Jime'nez et al., 2006).

Thus, some medical institutions recommend genetic testing for patients with VHL and SPA (Ali Tootee and Shirin Hasani-Ranjbar., 2012; Bausch et al., 2013; Andreasson et al., 2013). In order to find the relationship between VHL gene mutation type and the tumor type, we carried out VHL gene detection in a Chinese family with non-syndromic PCCs and patients with ASP. And now we present the result of VHL gene analysis.

## Materials and Methods

### *Families with non-syndromic PCCs*

The family with PCCs includes 56 members of 4 generations, and is from Gansu Province. Among them, 7 extended over 3 generations were diagnosed as PCCs. One died before admission and another died due to a cardiovascular incident. Five were initially evaluated; evaluation included medical history, physical examination, abdominal ultrasonography, computed tomography (CT) or magnetic resonance imaging (MRI). Laboratory tests included routine biochemical testing, blood catecholamines and 24h urinary catecholamine metabolite determination. The results showed that the levels of blood catecholamines and 24h urinary vanillylmandelic acid were elevated significantly (more than 3 times).

All 5 patients (proband, see Table 1) underwent surgical resection. The tumors were benign under pathological examinations. There were no recurrence and malignant transformation over the period of up to 23-year follow-up. Twenty members, including 5 live patients and 15 direct relatives, accepted genetic examination.

### *Patients with ASP*

Forty-one unrelated patients were selected from 91 with ASP who were registered in Urology Center of Lanzhou General Hospital, China, between 2005 and 2010, with the selection criteria (age below 60 yr, undertaking surgery resection and confirmed by pathology). They all participated in preoperative evaluation, including medical history, physical examination, abdominal ultrasonography, CT or MRI, routine biochemical testing, blood catecholamines and 24h urinary catecholamine metabolite determination. There were 17 males and 24 females (42.80±9.73 years in age); 15 cases had the left adrenal tumor, 24 cases with the right adrenal tumor, and 2 cases with bilateral adrenal tumors.

**Materials and PCR:** An informed consent was obtained from all participants. Genomic DNA analysis from peripheral blood leukocytes was conducted at the Urology Center of Lanzhou General Hospital, China for the 41 unrelated patients who underwent surgery for ASP and 20 members of the Chinese family. DNA extraction was performed by using D9081 kit (Takara Biotechnology, Dalian, China) according to the manufacturer's instruction. The three exons of the VHL gene were amplified and directly sequenced.

Primer sequences are available upon request. And the primers were synthesized by Takara. PCR amplification was carried out in 1×1 PCR buffer (Takara) that contained 400 m ol/liter deoxynucleotide triphosphate, 0.4 mol/liter of each primer, 2.5 units of Taq polymerase (Takara), and 100-200ng of DNA template in a 50µl volume. PCR conditions: one cycle of 15 min at 95°C; 30 cycles of 45 s at 95°C; 45 s at 63°C; 45 s at 72°C; followed by one cycle of 10 min at 72°C (Exon 1). one cycle of 15 min at 95°C; 30 cycles of 45 s at 95°C; 45 s at 59°C; 45 s at 72°C; followed by one cycle of 10 min at 72°C (Exon 2); one cycle of 15 min at 95°C; 30 cycles of 45 s at 95°C; 45 s at 57°C; 45 s at 72°C; followed by one cycle of 10 min at 72°C (Exon 3). The 7500 PCR System (Applied Biosystems, USA) was used for PCR reactions. The PCR products were performed sequence analysis by direct cycle sequencing using the Big Dye terminator v3.1 DNA Sequencing Kit (Applied Biosystems, USA) and an automated sequencer ABI PRISM 3730 (Applied Biosystems, USA). Mutations were confirmed by Taqman probe (the primers and probes were synthesized by Takara and checked by NCBI-BLAST).

## Results

VHL gene mutations, including 2 missense mutations and 1 insertion mutation, were identified in all 20 members of the Chinese family (5 patients and 15 relatives) as well as all patients with ASP. In the family, a disease-causing heterozygous H125P mutation was detected in the 5 probands with non-syndromic PCCs, whose onset age was between 8 and 20.

There were some members of this family also had H125P mutation of the VHL gene, but this proved to be a neutral variant and the levels of blood catecholamines and 24h urinary catecholamine metabolite were normal. One member (proband 1, male, 17 years old) was admitted to the hospital because of recurrent headaches, Palpitations, sweating and blurred vision of five month. Proband 1 had unilateral multiple PCCs. The carrier 1 (sister to proband 1) with bilateral PCCs, was accepted surgery, and the pathological result was benign. The presenting tumor types in probands and carriers from this family were non-symptomatic.

Out of the 41 unrelated patients with ASP, one patient had R120T missense mutation and 2 patients had 623 (^TTTGtG) insertion mutations, respectively. The proband 6 carrying R120T missense mutation had unilateral renal cyst and hypertension, whose family screening showed the presence of the same mutation in her two healthy relatives (carrier 9 and carrier 10, see Table 1), but her mother (carrier 8) also had unilateral PCC, whose outcome of pathology was benign.

There were no newly developed VHL-related tumors on the carrier 11 and carrier 12, carrying 623 (^TTTGtG) mutations. The proband 8 with unilateral PCC had bilateral renal cysts. His mother (carrier 13) with unilateral PCC was asymptomatic, refusing to accept surgery. The family pedigrees and mutation sequencings table were presented in our early study (Andreasson et al., 2013; Zinnamosca et al., 2013).

**Table 1. Summary of Clinical Data and Mutation Analysis Results**

Patient	gender	PCC (age at first diagnosis,years)	Other clinical presence	VHLD type	Mutation type	Mutation
<sup>a</sup> Proband1	Male	Unilateral multiple,18		IIC	Missense	H125P
<sup>a</sup> Carriers1(sister to proband 1)	Female	Bilateral,29		IIC	Missense	H125P
<sup>a</sup> Carriers2(nephew to proband 1)	Male		Asymptomatic		Missense	H125P
<sup>a</sup> Proband2	Male	Bilateral,8		IIC	Missense	H125P
<sup>a</sup> Proband3	Male	Bilateral,17		IIC	Missense	H125P
<sup>a</sup> Carriers3(son to proband 2)	Male		Asymptomatic		Missense	H125P
<sup>a</sup> Proband4	Female	Bilateral,16			Missense	H125P
<sup>a</sup> Carriers4(father to Proband3 )	Male		Asymptomatic		Missense	H125P
<sup>a</sup> Carriers5(brother2 to Proband3)	Male			IIC	Missense	H125P
<sup>a</sup> Carriers6(nephew to Proband3)	Male		Asymptomatic		Missense	H125P
<sup>a</sup> Carriers7(niece to Proband3)	Male		Asymptomatic		Missense	H125P
<sup>a</sup> Proband5	Male	Bilateral,16		IIC	Missense	H125P
<sup>b</sup> Proband6	Female	Unilateral,28		IIC	Missense	R120T
<sup>b</sup> Carriers8(mother to Proband6)	Female	Unilateral,54		IIC	Missense	R120T
<sup>b</sup> Carriers9(aunt to Proband6)	Female		Asymptomatic		Missense	R120T
<sup>b</sup> Carriers10(cousin to aunt)	Female		Asymptomatic		Missense	R120T
<sup>b</sup> Proband7	Male	Unilateral,22		IIC	Insertion	623(^TTTGTtG)
<sup>b</sup> Carriers11(father to Proband7)	Male		Asymptomatic		Insertion	623(^TTTGTtG)
<sup>b</sup> Carriers12(sister to Proband7)	Female		Asymptomatic		Insertion	623(^TTTGTtG)
<sup>b</sup> Proband8	Male	Unilateral,29	Bilateral Renal cysts	IIB	Insertion	623(^TTTGTtG)
<sup>b</sup> Carriers13(mother to Proband7)	Female	Unilatera,50		IIC	Insertion	623(^TTTGTtG)

<sup>a</sup>:members from a Chinese family with Non-syndromic PCCs; <sup>b</sup>:patients with ASP

## Discussion

In the present study, we reported that genetic abnormal origin of VHLD in members from the Chinese family with non-syndromic PCCs and in 3 of 41 patients with ASP (7.3%). We detected 3 disease-causing novel genetic mutations, included 2 missense mutations (H125P and R120T) and an insertion mutation (623(^TTTGTtG)), located in exon 2 encoding  $\beta$ -domain of pVHL. Mutations resulted in dysfunction pVHL and were associated mainly with VHL type 2B or type 2C. Some VHL mutations of RCC have been reported in Chinese studies (Zinnamosca et al.,2013; Kim et al., 2013; Qi et al.,2013; Mete et al., 2013; Chen et al., 2013). These mutations in VHL patients differ from those observed in domestic VHLD kindreds. The reports about missense mutations and insertion mutations in Chinese with VHLD are first to report (Zhou et al., 2007; Zhang et al., 2009).

The frequency of PCC in VHLD patients was about 25%. But the penetrance in members from the Chinese family in association with H125P (12.5%) was lower than the other familial pheochromocytomas (Lanikova et al., 2013). The penetrance in patients with ASP (7.3%) was similar to the figure observed in Western, Japanese, and Chinese VHLD kindreds (Tootee and Hasani-Ranjbar,2012; Vaganovs et al., 2013; King and Pacak, 2013). The age of tumor onset was between 5 and 20 associated with H125P. And most patients with H125P were with bilateral PCCs. The age of tumor presentation was between 22 and 54 associated with R120T and 623(^TTTGT tG). The patients with R120T and 623(^TTTGTtG) had unilateral PCC. There were similar results of biochemical testing in patients with mutations, of which the levels of blood catecholamine and 24h urinary catecholamine metabolite were all increased

significantly (compared with normal level). We also tested healthy members and found that the mutations were absent in all clinical healthy members from the Chinese family who participated genetic screening and some direct relatives of patients with ASP. Limited participation in genetic evaluation among carriers and their relatives who have no abnormal clinical findings limited our ability to detect genetic modifiers and environmental factors.

According to guidelines from JCEM of 2006, genetic examinations for ASP are recommended for individuals with familiar history of PCCs especially for those of which a family member was diagnosed with PCCs under the age of 20 years, the PCCs were bilateral or in case of an extra-adrenal pheochromocytoma with catecholamine synthesis. Evidence indicates. Testings play an important role in medical management, such as timely diagnosis of non-syndromic PCCs, accurate genetic counseling and predictive testing, tumor resection, postoperative care, and close follow-up based on gene-specific clinical protocols. For the purposes of genetic counseling and genetic screening, we need to underscore the importance of several demographic and clinical features associated with the presence of a germ line mutation, including age at diagnosis, multifocal disease, extra-adrenal location, and malignant tumors. According to the present study and reference, individuals whose age at onset were less than 50 years and had a family history, non-syndromic suspected pheochromocytoma, ganglion cell tumor syndrome group and pheochromocytoma, should accept VHL gene detecting. These individuals and their relatives could benefit from genetic detecting technology immediately.

As the sequencing technology progresses, genetic testing cost is decreasing. According to our method, the cost for searching for VHL mutations (covering 3 exons) by sequencing is 216 (RMB yuan), including DNA

extraction and PCR-sequencing of each exon on DNA strands. Our goal in further research is to find new lab tech that is more sensitive with low cost and more cases of mutations will be found.

In conclusion, in our study we identified the VHL gene mutations (H125P, 623(ˆTTTGTtG) and R120T) in all individuals with PCCs who are members of a single Chinese family as well as three patients with APS in a cohort of 41. These mutations resulted in dysfunction of pVHL. The genetic analysis is helpful for medical management of patients with HVLD or ASP and exploration of VHLD pathogenesis .

## Acknowledgements

We thank Dr. Ha Xiao-qin and her team for the helpful advice and excellent technical assistance.

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