Identification of Somatic Mutations in the von Hippel–Lindau (VHL) Gene in a Patient With Renal Cell Carcinoma

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One of the known causal molecular events in renal cell carcinoma is somatic mutation in the von Hippel–Lindau (VHL) gene. Our study describes a 51-year-old Taiwanese man who had bilateral renal cell carcinoma. The patient underwent radical nephrectomy without postoperative chemotherapy or radiotherapy, and is still alive after renal transplantation without tumor recurrence after > 5 years. To clarify his predisposition for bilateral tumors, we performed molecular genetic analysis of the VHL gene in this study. Polymerase chain reaction–single-strand conformation polymorphism and direct sequencing were performed on DNA of blood samples and paraffin-embedded tumor specimens from this patient. DNA from peripheral blood lymphocytes tested negative for germline mutations. However, there were two heterozygous alleles in the promoter and 3' untranslated regions of this gene. Nonetheless, the DNA from his tumors showed loss of heterozygosity (LOH) in these two loci. In addition to the LOH, we identified some different somatic mutations in his tumor tissues: C287T and G460A in the right-sided tumor, and G244A and G390A in the left-sided tumor. The possible roles of these genetic polymorphisms and point mutations in his renal tumorigenesis are discussed. This report provides new insights into renal cell carcinoma that result from VHL gene alterations in Taiwan. [J Formos Med Assoc 2009;108(11):886–893]

Key Words: mutation, renal cell carcinoma, Taiwan, von Hippel-Lindau gene
deletions of the VHL gene have been observed in many cases of sporadic clear cell RCC, in addition to families with VHL syndrome.\textsuperscript{8,9} VHL gene inactivation by promoter hypermethylation also has been detected in some sporadic cases of RCC.\textsuperscript{10}

Hundreds of mutations have been described in the VHL gene to date (Human Gene Mutations Database; HGMD).\textsuperscript{11} The spectrum of VHL gene mutations is enormous and there is a significant difference between somatic and germline mutations.\textsuperscript{12} Many countries have established national test groups to improve the diagnosis and treatment of individuals with VHL syndrome.\textsuperscript{4} The relation between VHL gene inactivation and sporadic clear cell RCC also has been noted in Japanese and Chinese populations.\textsuperscript{13,14}

In the present study, a 51-year-old man was found to have multifocal RCC and underwent bilateral nephrectomy. Histological examination revealed that these tumors were the clear-cell type. We performed genetic analysis to clarify the role of the VHL gene in the renal tumorigenesis of this patient.

**Case Report**

A 51-year-old man had RCC that arose from bilateral renal cysts, which was confirmed as the clear-cell type by histopathological examination. He was treated with bilateral nephrectomy and subsequent renal transplantation. He had seven siblings and two children. After informed consent, a detailed family history was obtained and blood samples were obtained from the patient and several of his family members. Paraffin-embedded tumor tissue samples were provided by the Department of Pathology, Lo-Pung Poh-Ai Hospital, Northeastern Taiwan. Histopathological examination revealed multicentric, bilateral clear cell renal carcinoma. A large tumor (3.5 × 3.3 × 3.1 cm) and four small tumor nodules (1.2 × 1.0 × 0.8 cm) were noted in the right kidney. The renal capsule and surrounding fatty tissue were not affected by the tumor. Cut sections of the left kidney showed a tumor mass of 5.7 × 5.3 × 5.2 cm, and two tumor nodules that measured up to 2.1 × 1.6 × 1.5 cm. The tumor nodules were confined mostly within the renal capsule, and microscopically invaded the nearby perirenal fat tissue. Approval to perform the study was obtained from the Institutional Review Board at the Chung Shan Medical University Hospital, Taichung, Taiwan.

Genomic DNA was isolated from peripheral blood lymphocytes using the QIAamp DNA Blood Kit (QIAGEN GmbH, Hilden, Germany). DNA was also prepared from paraffin-embedded tissue sections using the QIAamp Tissue Kit (QIAGEN GmbH). DNA concentration of each sample was measured using spectrophotometry and adjusted to 100 ng/μL for the subsequent analyses. The three exons of the VHL gene were amplified in seven overlapping fragments (Figure 1) using published primers and experimental conditions.\textsuperscript{11} Fragment 1 amplified the promoter and 5′-untranslated region (UTR), along with the first decadal codons. Fragments 2 and 3 amplified the

![Figure 1](image-url)

**Figure 1.** Distribution of the mutations identified within the VHL gene in a Taiwanese patient with renal cell carcinoma. The three exons of the VHL gene with nucleotide numbers are indicated (GenBank accession number NM_000551). Polymerase chain reaction fragments, shown by numbers 1–7, were generated using primer sequences reported previously.\textsuperscript{11} The short arrowheads indicate the positions of nucleotide substitutions in this study. P = promoter; UTR = untranslated region.
regions of exon 1 and 2, respectively. Fragments 4 and 5 amplified the overlapping regions of exon 3. The 3’-UTR was analyzed by the overlapping fragments 5–7. Each PCR fragment was amplified using GeneAmp PCR system 2400 (Perkin Elmer Foster City, CA, USA), followed by single-strand conformation polymorphism (SSCP) analysis. The PCR products were run using GenePhor™ DNA Separation System (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for 55 minutes at 600 V and at two different temperatures (12°C and 23°C). The single strands were visualized using the PlusOne™ DNA Silver Staining Kit (Amersham Pharmacia Biotech). Samples from SSCP analysis were re-amplified and the PCR products were purified using QIAquick PCR Purification Kits (QIAGEN GmbH) for cycle sequencing. Cycle sequencing with fluorescently-labeled dideoxy chain terminators (ABI Prism Kit; Applied Biosystems, Taipei, Taiwan) was performed according to the manufacturer’s protocol. The sequencing primers were the same as those for the preceding PCR. When a mutation was detected, the nucleotide sequence was confirmed on both strands.

Mutations in the \( VHL \) gene are associated closely with the clear-cell type of RCC\(^{15} \). Therefore, we hypothesized that this patient carried an inactivated form of the \( VHL \) gene in his genome. First, we used long-range PCR to detect potential deletions in the \( VHL \) gene,\(^{16} \) and found no difference in the long PCR products, using genomic DNA extracted from the patient and his family members, as compared with a normal unrelated individual (data not shown). Thus, it is likely that this patient did not carry a germline gene rearrangement or any deletion in his \( VHL \) gene. Next, to identify a germline mutation in the \( VHL \) gene, we amplified the seven overlapping fragments (Figure 1) and performed SSCP analysis using genomic DNA isolated from lymphocytes of the patient and his family members. We found no difference among these samples (data not shown). We confirmed these results by directly sequencing all the PCR products, and verified that the \( VHL \) gene was not mutated in the lymphocytic DNA from this patient. The results indicated that no \( VHL \) germline mutation was present in this case.

More importantly, we identified two heterozygous loci in the \( VHL \) gene in his lymphocytic DNA. Both were allelic single nucleotide polymorphisms (SNPs) with an A or a G, which were identified previously by Payne et al in 1994 and Geil et al in 1998, respectively.\(^{17,18} \) When blasted against the reference sequence of GenBank (accession number NM_000551), one SNP was located at nucleotide 19 (19 SNP) within the promoter region of the \( VHL \) gene (Figure 2A), and the other SNP was located in the 3’-UTR at nucleotide 1149 (1149 SNP) (Figure 2B). Haplotype analysis of these two SNPs was performed by sequencing the genomic DNA from lymphocytes of the siblings of the proband and their offspring. The results indicated that the two A alleles in the 19 SNP and 1149 SNP were on the same chromosome as the two Gs on the other chromosome in his family members (data not shown).

We also analyzed the tumors from this patient for the presence of somatic gene alterations. Loss of heterozygosity (LOH) in the above two SNPs was found in the tumors. In the right-sided tumor, the 19 SNP was not altered, whereas the 1149 SNP showed alteration of the heterozygous pair in the A allele only (Figures 2C and 2D). That is, there were two alleles in the 19 SNP and only one allele in the 1149 SNP. It is possible that part of the \( VHL \) gene, downstream of 19 SNP, including the 1149 G allele, was deleted, or the 1149 G allele of the \( VHL \) gene on one chromosome was mutated. In the left tumor, the two heterozygous alleles became homozygous. There was only one allele in the 19 SNP or 1149 SNP. The 19 SNP changed to the G allele and the 1149 SNP changed to the opposite A allele (Figures 2E and 2F). This suggests that recombination happened between the 19 and 1149 SNP on one chromosome, followed by the loss on the other chromosome.

In addition to the LOH on the SNPs, we also identified some different \( VHL \) somatic mutations in the tumor tissue at both locations. In the right-sided tumor, sequence analysis revealed two missense mutations, compared with the blood
Figure 2. Partial sequencing chromatograms represent the genetic profiles of fragment 1 (A, C and E) and fragment 6 (B, D and F) in forward directions from the blood (A and B), the right-sided tumor (C and D), and left-sided tumor (E and F). The 19 SNP in fragment 1 and 1149 SNP in fragment 6 are marked with the rectangular frame.
Figures 3A and 3B, at nucleotides 287 and 460. The former mutation showed that one allele was a C to T transition (Figure 3C) that resulted in a proline to a leucine substitution (P25L). The latter mutation revealed heterozygosity in two alleles at nucleotide 460 (Figure 3D). On one chromosome, a G to A transition led to the substitution of valine to isoleucine at codon 83 (V83I) on the affected allele. The other chromosome showed the original G nucleotide at position 460. In the left-sided tumor, sequence analysis revealed two mutations at nucleotide 244 and 390 by comparison with the blood (Figures 4A and 4B). Both mutations showed that only one allele resulted in a G to A transition (Figures 4C and 4D). The former was a missense mutation that changed the amino acid encoded at codon 11 from alanine to threonine (A11T). The latter was a silent mutation that did not change the amino acid residue at codon 59, and it destroyed a recognition sequence of the restriction endonuclease NotI.

To summarize, no germline mutation was present. In the right-sided tumor, the missense mutation at nucleotide 287 destroyed both alleles. One of them had an additional missense mutation at nucleotide 460. In the left-sided tumor, one copy of the VHL gene was lost. One copy had a missense mutation at nucleotide 244 and a silent mutation at nucleotide 390.
Discussion

In the present study, we concluded that there was no germline mutation in the patient and his family members, based on the results from SSCP analysis and DNA sequencing. However, we found that this patient and his siblings carried two heterozygous alleles on the 19 and 1149 SNPs in the VHL gene. These SNPs could represent novel polymorphisms, and are important in RCC tumorigenesis. In this patient, LOH was detected only on 1149 SNPs in the right-sided tumors, but was found on both SNPs in the left-sided tumors. According to the pathology results from the patient, the left-sided tumor was more aggressive than the right-sided tumor. In the same genetic matrix, we speculate that the extent of somatic genetic alteration of those two SNPs was correlated with the severity of the tumors. Therefore, the data suggest that these genetic polymorphisms are a susceptibility or risk factor for VHL-related tumors.

The VHL gene behaves as a typical tumor suppressor, as defined in Knudson’s theory of human carcinogenesis. The sporadic tumors in our patient were predicted from a two-hit model and late onset. In the left-sided tumor, recombination and heterozygous allele loss of VHL gene served as one hit, and the somatic mutation, G244A (amino acid change A11T) on the other chromosome served as the other hit. The loss of the normal VHL allele was likely to have been an early event in tumor development, as suggested by Lubensky et al. We found two somatic mutations, C287T and G460A, in the DNA of the right-sided tumor. The G460A mutation, V83I, inside the mutation cluster region 1 and the β protein-binding domain have been identified previously.

Figure 4. Partial sequencing chromatograms represent the genetic profiles of fragment 2 in forward directions from the blood (A and B) and left-sided tumor (C and D). The nucleotides 244 (A and C) and 390 (B and D) are marked with the rectangular frame.
Similarly, the C287T mutation, P25L, in the right-sided tumor also has been reported previously in the HGMD (accession number CM981994), and is in the acidic pentameric repeat. Our results suggest that these mutations serve as the two hits and are pathogenic. The mutations of A11T in the left-sided tumor and P25L in the right-sided tumor were located before the second methionine start codon, which suggests that codons 1–54 before the second start site are required for tumor suppressor function.

In the DNA from the right-sided tumor of this patient, the C287T mutation and 1149 SNP in the VHL gene were homozygous. However, the two alleles of the 19 SNP and G460A mutation were heterozygous. A large tumor and four small tumor nodules were noted in the right-sided tumor, therefore, equal peaks of A and G were observed, which suggested possible heterogeneity.

More interestingly, similar cases of patients with multiple mutations inside the tumors have been described previously in conventional RCC patients. Furthermore, in our patient, the mutation status differed between the right- and left-sided tumors, which suggested two distinct primary origins. The concurrence of these mutations in a patient indicates that one or more modulator gene(s) might exist. It has been suggested that genetic instability is a detectable phenomenon in human RCC, and that it is associated with the development of the disease.

The characteristics of VHL RCC include multifocal and bilateral tumors, cystic organization and low-grade histology. All these characteristics were observed in our index patient. We conclude that variants of the VHL gene played an important role in this case. They appear to predispose an individual to the development of renal lesions. Some mutations or polymorphisms also affect the prognosis of clear-cell type RCC. Thus, the identification of genetic changes can improve the clinical diagnosis. After bilateral nephrectomy and renal transplantation, this patient is still alive without tumor recurrence after >5 years, which suggests that these are not highly aggressive tumorous cells. We could consider these genetic variants to be favorable prognostic factors. This might become a significant consideration in individualized treatment for RCC in the future.

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References

factor for VHL patients to develop an RCC. *Hum Mutat* 1999;13:464–75.


