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(Citation)
The Kobe journal of the medical sciences,46(4):147-153

(Issue Date)
2000-08

(Resource Type)
departmental bulletin paper

(Version)
Version of Record

(URL)
https://hdl.handle.net/20.500.14094/E0001054

Kobe J. Med. Sci. 46, 147/153, August 2000

MOLECULAR GENETIC ANALYSIS AND MUTATION SCREENING OF THE VHL GENE IN A JAPANESE FAMILY WITH VON HIPPEL-LINDAU DISEASE

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KEY WORDS

von Hippel-Lindau disease; the VHL gene; tumor-suppressor gene germline mutation; mutation screening

ABSTRACT

Von Hippel-Lindau (VHL) disease is an autosomal dominantly inherited familial cancer syndrome which predisposes individuals to a variety of malignant and benign tumors. Its penetrance is almost 100% by 61-70 years of age. We have identified a germline mutation in the VHL gene of a Japanese male patient with a retinal hemangioma in the left eye, an intracranial hemangioblastoma, a left renal tumor and bilateral pheochromocytomas. Screening for the mutation was performed in all members of the patient's family at risk for VHL disease. The mutation identified in the patient was a missense mutation at codon 238 (CGG to CAG). The elder daughter, who did not show any clinical symptoms, was found to carry the same mutation. In order to detect and treat tumors in VHL patients at an earlier stage, it is necessary to identify and screen for the VHL gene mutation in all family members known at risk.

Received for publication: January 17, 2000

INTRODUCTION

Von Hippel-Lindau (VHL) disease is an autosomal dominantly inherited familial cancer syndrome which predisposes sufferers to a variety of benign and malignant tumors. The most common tumors found in patients with VHL disease are retinal and central nervous system (CNS) hemangioblastomas, renal cell carcinoma, and pheochromocytoma. The incidence of VHL is estimated at three cases per 36,000, and penetrance is 99% by 61-70 years of age[7]. VHL patients have been classified into three types based on the clustering mode of the tumors: patients with renal cell carcinoma but without pheochromocytoma (type 1), patients with pheochromocytoma, but without renal cell carcinoma (type 2A), and patients with pheochromocytoma and with renal cell carcinoma (type 2B)[8].

The VHL gene was mapped to chromosome 3p25- p26 region by linkage analysis[10] and was isolated by Latif et al.(1993)[5] by positional cloning. The gene is composed of three exons and encodes a protein consisting of 213 amino acids. The VHL gene is now thought to be one of the tumor suppressor genes; the exact biochemical function of the VHL protein is unknown, but the most important clinical complications stem from the role of the VHL protein in tumor suppression[6]. Knudson's two-hit hypothesis explains tumorigenesis by the inactivation of both alleles of a gene which had once prevented the tumor[3,4]. In tumor-prone genetic diseases such as VHL disease, even if one abnormal allele of the VHL gene is present as a germline mutation, the remaining normal allele is usually sufficient to prevent tumor formation. A subsequent somatic mutation then inactivates the second allele, allowing the tumor to form[9].

Cloning of the VHL gene has led to the identification of germline mutations in VHL disease patients throughout the world[13] and germline mutations have now been detected in 70-95% of VHL families[8]. A total of 146 germline mutations of the VHL gene has been reported so far but this figure is still increasing[8]. A wide variety of germline mutations include missense, nonsense, frameshift deletions or insertions, in-frame deletions and splice site mutations causing splicing defects[2,8,13]. Genomic DNA analysis of Japanese patients with VHL disease revealed essentially similar findings to those observed in Western countries[11].

In this study, we identified a germline mutation in the VHL gene of a Japanese patient with intracranial hemangioblastoma, a renal tumor and pheochromocytoma (VHL type 2B), and carried out screening for the mutation in all family members known to be at risk. We identified a missense mutation at codon 238 (CGG to CAG, arginine to glutamine) in the patient and his asymptomatic daughter.

MATERIALS AND METHODS

Patient

The patient was a 29-year-old Japanese male. He developed retinal hemangioma in the

left eye at 11-years of age, and underwent laser surgery. When he was 29-year-old, he suffered from hypertension and blurred vision in his right eye. He was referred to Himeji Red Cross Hospital and diagnosed as having VHL type 2B because of multiple tumors including a left renal tumor and bilateral adrenal pheochromocytomas. Neurological studies showed the presence of an intracranial hemangioblastoma. His mother, brother and two daughters (2- and 5-year-old) had no clinical symptoms. His father died suddenly at 63-years of age but an autopsy was not performed. Molecular genetic analysis of the patient, his mother and brother was performed after obtaining their informed consent. Informed consent for genetic of the two daughters, was obtained from their parents.

Polymerase chain reaction (PCR) and direct sequencing analysis

Genomic DNA was extracted from peripheral blood leukocytes with a Puregen DNA Isolation Kit (Gentra Systems, Inc., MN, USA) and used as a template for PCR. To detect a mutation in the VHL gene, the PCR was carried out as described by Crossey et al[2] with some modifications.

Two primer sets were used to amplify VHL exon 3: primer set A (5'-CCTTGTACTGAGACCCTAGTCTGCCACT-3' and 5'-CAAGACTCATCAGTACCA TCAAAAGCTG-3') for the sequence encompassing nucleotides 677 -915 and set B (5'-GCGATGCCTCCAGGTTGTCC-3' and 5'-TGCCCCTAAACATCACAATGCCTA-3') for the sequence encompassing nucleotides 693 -1036. Amplification of VHL exon 3 was done in a thermal cycler (Program Temp Control System PC-700; Astec, Fukuoka, Japan) in 30 μl volume reactions containing 300 ng of genomic DNA in 1 x GC Buffer I (Takara Shuzo Co., Ltd. Biomedical Group, Shiga, Japan) with 2.5 mM MgCl2, 250 μM dNTPs, 0.3 μM primers and 2.5 units of TaKaRa LA Taq™ (Takara Shuzo Co., Ltd. Biomedical Group). The temperature conditions for PCR included an initial denaturation at 94°C for 7 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 63°C for 1 min, extension at 72°C for 3 min, and final extension at 72°C for 7 min. PCR-amplified fragments were electrophoresed in 3% agarose gels and photographed after ethidium bromide staining. The expected sizes of the two PCR-amplified fragments using primer sets A and B were 291 bp and 343 bp, respectively.

PCR-amplified fragments were purified using the GENECLEAN®II Kit (BIO 101 Inc. La Jolla, CA, USA) and sequenced directly. The sequencing reaction of the purified PCR-amplified fragments was performed using a dye-terminator cycle-sequencing kit (ABI PRISM 310; Perkin-Elmer Applied Biosystems, Foster City, CA, USA) according to the supplier's instructions. The sequencing reaction products were electrophoresed on a genetic analyzer (ABI PRISM 310; Perkin-Elmer Applied Biosystems) and then analyzed using the sequencing analysis software (ABI PRISM 310; Perkin-Elmer Applied Biosystems).

Mutation screening (PCR and restriction enzyme digestion analysis)

The PCR-amplified fragment with primer set A (291 bp) was digested with restriction

enzyme Mspl. Restriction fragments were separated by 3% agarose gel electrophoresis and photographed after ethidium bromide staining. When there was no missense mutation at codon 238 of the VHL gene, the PCR-amplified product with primer set A was digested completely into two fragments of 203 bp and 88 bp.

Nucleotides were numbered according to the cDNA sequence published by Latif et al[5].

RESULTS

PCR and direct sequencing analysis

PCR and direct sequencing analysis of the VHL gene disclosed heterozygosity of the wild and mutated alleles in the patient. The mutated alleles carried a transition, G to A at nucleotide 713, which was to be a missense mutation, CGG to CAG, at codon 238 (Fig. 1). Here we adopted "codon 238" as a widely accepted codon number, although this codon has been denoted as "codon 167" by Zbar et al[13]. This missense mutation causes an amino acid substitution, arginine to glutamine.

Mutation screening

Mutation screening was perfomed on all family members. A combination of PCR and restriction enzyme digestion methods were used for the identification of the mutation at codon 238, because of the presence of a natural restriction enzyme site for Mspl at codon 238. The mutation at codon 238 would disrupt the Mspl site. When the mutation was present, the PCR-amplified product with primer set A was not digested and remained 291 bp in size. After Mspl digestion, the patient and his elder daughter (5- year- old) presented three bands of 291, 203 and 88 bp, whereas the other members of the family showed only the 203 and 88 bp bands (Fig. 2). The elder daughter was diagnosed as having a mutation at codon 238 in the VHL gene.

To confirm the presence of the missense mutation in the elder daughter, PCR and direct sequencing analysis were performed. Sequencing data showed the same missense mutation as in her father, CGG to CAG, at codon 238 in one allele. Thus, the daughter was proved to be heterozygous for the wild and mutated alleles of the VHL gene.

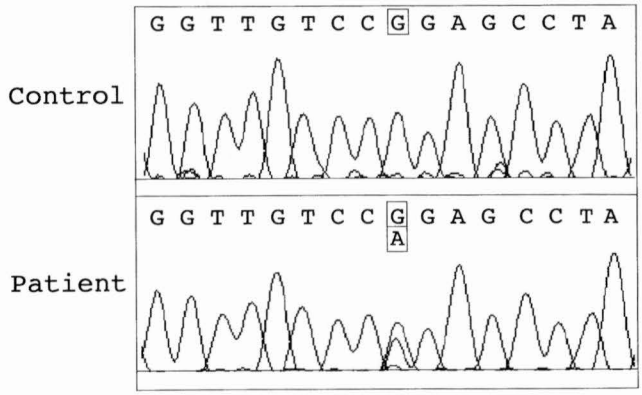

Fig. 1. *PCR and direct - sequencing analysis of the patient.*
The mutated allele of the VHL gene carried a transition, G to A at nucleotide 713, which was to be a missense mutation, CGG to CAG, at codon 238.

Fig. 2. *PCR and restriction enzyme digestion analysis.*
The identified mutation disrupted the Msp1 site at codon 238 of the VHL gene. When the mutation was present, the PCR-amplified product was not digested and remained 291 bp in size. When the mutation was absent, the PCR amplified product was completely digested into two fragment of 203 bp and 88 bp (the 88 bp-band was too faint to be visualized on the photograph). After Msp1 digestion, the patient and his 5-year-old daughter both presented a band of 291 bp. Thus, the elder daughter was diagnosed as having a mutation at codon 238 in the VHL gene, like her father.

DISCUSSION

The genotype-phenotype correlations in VHL disease is currently being clarified. In our patient with VHL type 2B, who developed bilateral pheochromocytomas, a missense mutation was identified in codon 238, leading to an amino acid substitution, arginine to glutamine. Missense mutations at codon 238 have been reported to convey a high risk of pheochromocytoma[1,2,13]. According to Zbar et al[13], 21 of 33 families with mutations at codon 238 had pheochromocytomas compared to 15 of 223 families without a mutation at this codon. Our findings also supported the results reported by Zbar et al.

The identification of germline VHL gene mutations improves the management of VHL families by allowing accurate pre-clinical diagnosis and risk assessment. We performed mutation screening on all members of the patient's family. One of his two daughters was demonstrated to have a mutated allele in the VHL gene by PCR and restriction enzyme digestion analysis, and this was confirmed by direct-sequencing analysis. This patient should be followed-up closely, because the penetrance of VHL is almost 100% by age 61-70 years of age[7]. An objection from the ethical principal of autonomy may be raised to our mutation screening or identification of the children: it is appropriate and even essential that the children be informed of their at-risk status upon reaching the age of reason. However, CNS, retinal and other visceral tumors of VHL disease may develop during childhood[12]. Indeed this patient may go on to develop tumors as her father had retinal hemangioma at 11 years of age. Mutation identification or screening enables closer follow-up of the family members at risk, and closer follow-up enhances the detection of early-stage tumors.

In conclusion, a mutation at codon 238 of the VHL gene was identified in a patient with VHL type 2B, and the family members at risk of VHL disease were screened for the same mutation. In order to detect and treat tumors at an earlier stage, it is necessary to identify and screen for the mutation in the VHL gene in all family members known to be at risk.

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