Detection of germline mutations in the von Hippel-Lindau disease gene by the primer specified restriction map modification method

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Abstract

Von Hippel-Lindau disease (VHL) is an inherited disorder characterised by a predisposition to develop tumours in the eyes, central nervous system, kidneys, and adrenal glands. Recently the VHL gene was cloned and shown to be mutated in 75% of US and Canadian VHL families. To develop simple, rapid methods for the detection of mutations found in large numbers of affected people, we designed tests based on the primer specified restriction site modification method. These tests have proved useful in identifying asymptomatic mutated VHL gene carriers who have the nt 505 T to C mutation or the nt 686 T to C mutation. Together with an *MspI* digestion test which can detect a mutation hot spot in codon 238, polymerase chain reaction/restriction endonuclease based tests can now detect VHL mutations in more than 50% of VHL type 2 families.

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Von Hippel-Lindau disease (VHL) is an autosomal dominantly inherited neoplastic disorder characterised by a predisposition to develop retinal angiomas, cerebellar haemangioblastomas, pheochromocytomas, renal cell carcinomas, pancreatic cysts, and epididymal cysts. Clinical screening for early detection of eye, brain, and kidney tumours has been recommended.^{1,2} The detailed genetic mapping of the VHL disease gene to chromosome 3p25-26³⁻⁸ enabled presymptomatic diagnosis of VHL disease by linkage analysis in some families.^{9,10} Recently, the VHL tumour suppressor gene was isolated by positional cloning methods.¹¹ It is now feasible to identify specific mutations in affected subjects and to follow these mutations in families. So far, germline mutations of the VHL gene have been described in families from the USA,^{12,13} England,¹⁴ Germany,¹⁵ and Japan (T Shuin, personal communication). The spectrum of mutations in the VHL gene among these different families is diverse including 80 different point mutations, small deletions/insertions, and large deletions (4-380 kb).¹²⁻¹⁵ Thus, identification of an unknown mutation requires several specialised, time consuming methods such as Southern blotting, pulsed field gel electrophoresis, or single strand con-

formational polymorphism analysis (SSCP) and DNA sequencing.

Previously we detected 85 different mutations in 114 VHL families and could identify genotype-phenotype relationship in these families.¹² The types of mutations responsible for VHL without pheochromocytoma (VHL type 1) differed from those responsible for VHL with pheochromocytoma (VHL type 2). Fifty-six percent of the mutations responsible for VHL type 1 were microdeletions/insertions, nonsense mutations, or large deletions (4-380 kb); 96% of the mutations responsible for VHL type 2 were missense mutations.¹²

The VHL families with point mutations that generated or destroyed unique restriction sites could be further tested for asymptomatic gene carriers by restriction enzyme digestion of polymerase chain reaction (PCR) products. However, point mutations in several families did not result in changes of restriction sites. Nt 505 T to C and nt 686 T to C were among the mutations which did not change a restriction site. Nt 505 T to C was found in 14 VHL families in the Black Forest area of Germany¹⁵ and two families in the USA.¹² Mutation at nt 686 T to C was found in two large families in the USA.¹² There were more than 100 patients with the 505 mutation and more than 75 patients with the 686 mutation. Many asymptomatic relatives in these families require genetic testing to determine whether they carried the mutant VHL gene. Haliassos *et al*¹⁶ introduced the primer specified restriction map modification method for analysis of a point mutation in the Kirsten ras oncogene that did not require restriction site changes. Point mutations in cystic fibrosis, retinitis pigmentosa, cholesteryl ester transfer protein deficiency, multiple endocrine neoplasia (MEN) type 2A, and familial medullary thyroid carcinoma have been analysed with this method.¹⁷⁻²⁰ In this report we used this method to detect the nt 505 or the nt 686 change in the VHL gene and could confirm its usefulness in testing a large number of VHL patients.

Methods

FAMILIES

We tested two large VHL families with point mutations at nt 505 T to C (family 3127) or nt 686 T to C (family 4475). Both mutations were found by SSCP and DNA sequencing previously.¹² No restriction site changes were available to detect these mutations. Forty-seven members were affected in family 3127 and we

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have 10 DNA samples including six clinically diagnosed VHL patients. Family 4475 had 51 affected members and we tested four affected patients who are fairly distant from each other in the pedigree.²² In our previous screening of 114 VHL families we found another family with the same change at nt 505 (family 3476) and another family with the same change at nt 686 (family 4408).

PCR WITH MODIFIED PRIMERS AND RESTRICTION ENZYME DIGESTION

To create the available restriction site change in the mutation region, we designed modified primers for the PCR reaction. For the nt 505 T to C mutation the primer TK 1 (5'-TGCCAGGCGGCAGCGTTGGAT-3') and the primer MA2A (5'-GGCCCGTGCTGC-GCTCGGTGAAC-3') amplify 135 bp PCR product. The primer TK 1 has a mismatch (underlined) with the genomic sequence and creates an *FokI* site in the mutant allele of the PCR product. For the nt 686 T to C substitution the primer TK 3 (5'-TCCGGA-CAACCTGGAGGCATCGCTCTTGC-3') and the primer YH1A (5'-TTCCTTGTAC-TGAGACCCTAGT-3') amplified 98 bp fragments. The primer TK 3 contains a mismatch (underlined) which provides an *AciI* site only in a mutant allele. PCR was performed with an initial three minutes' denaturation at 95°C followed by 30 cycles of 45 seconds at each of 95°C, 59°C, 72°C, and final extension for five minutes at 72°C in a PCR 9600 Perkin Elmer Cetus machine. PCR products were ethanol precipitated then dissolved in 10 µl of TE; 10 units of *FokI* or *AciI* (New England Biolabs) and 1.1 µl of 10 × buffer supplied by the manufacturer were added and incubated at 37°C for three hours. The digested products were electrophoresed in a 2% TBE agarose gel and visualised by ethidium bromide staining.

Results

In the previous study we detected two point mutations in the VHL gene that did not create or destroy a restriction site. As these mutations were found in large families that contained many at risk asymptomatic relatives, we created simple methods to test these mutations. One mutation was a T to C substitution at nt 505 found in families 3127 and 3476; the other was a T to C substitution at nt 686 found in families 4475 and 4408. DNA samples from families 3127 and 3476 were amplified by PCR with primers TK1 and MA2A, then the 135 base pair PCR product was digested by *FokI*. Affected patients in both families showed a 104 bp fragment originating from the nt 505 T to C mutation (figure A,B). One unaffected member in family 3476 (patient II-3) and three asymptomatic young sibs in family 3127 (II-5, 7, 8) showed the 104 bp fragment. Patient II-3 in family 3476 was considered to be an obligate gene carrier because of the family history. The three asymptomatic children who carry the mutant gene need clinical screening. For detection of the mutation at nt 686 T to C

substitution, we tested affected patients from family 4475 and 4408. DNA samples from these families were PCR amplified with primers TK3 and YH1A and then digested by *AciI*. Only the mutant allele created 68 bp fragment in addition to 98 bp PCR product in all affected subjects (figure C).

Discussion

Many genetic diseases can be diagnosed by identification of mutations in the gene responsible. Cystic fibrosis, neurofibromatosis, muscular dystrophy, and MEN type 2A are examples of hereditary diseases that can be subjected to DNA diagnosis. When the changes in the gene are a single base substitution or small deletion/insertion that create or destroy a unique restriction site, the presence of a mutation can be detected by digestion of the PCR product with a specific restriction enzyme. We tested 20 VHL families with this conventional restriction enzyme test and reconfirmed the identified mutation (data not shown). In cases of mutations that do not change any restriction site, primer specified restriction site modification makes it possible to detect changes easily with restriction enzymes. We used this method to detect two point mutations found in two large VHL kindreds and could confirm the mutation in affected patients as well as asymptomatic mutant gene carriers. The other members of these families are being screened with this method at other clinical laboratories.

In MEN type 2A, 84% of the causative mutation in the RET gene was in codon 634.²¹ In this case it was possible to screen the unknown genetic change in new MEN type 2A families by PCR amplification of the region followed by digestion with the appropriate restriction enzyme.^{19,20} In VHL, although the types of mutation vary from large deletions (4–380 kb) to many different point mutations, in some situations tests with restriction enzymes are still useful for detecting unknown mutations in new families. VHL families can be classified into two types based on the clinical heterogeneity and presence or absence of pheochromocytoma.^{22–24} VHL type 2 was defined by the presence of pheochromocytoma and 96% of the mutations responsible for the VHL type 2 were missense mutations including frequent mutations at codon 238.¹² This mutational hot spot at codon 238 (C to T substitution at nt 712 and G to A at nt 713) has been reported from several investigators from the USA,¹² England,¹⁴ and Japan (T Shuin, personal communication), and was strongly correlated with VHL type 2. In our previous study 10/23 (43%) of VHL type 2 families had this hot spot mutation.¹² These two mutations found in codon 238 were detected easily by a restriction enzyme test because they destroyed an *MspI* site. Those two families with nt 505 mutation and one of the families with nt 686 mutation were also classified as VHL type 2. Therefore, in 13 out of 23 VHL type 2 families (57%) we were able to detect their mutations by PCR-restriction enzyme based

Detection of germline mutations by primer specified restriction map modification. (A, B). Detection of nt 505 T to C mutation. DNA from members of family 3476(A) and 3127(B) were PCR amplified with primers TK1 and MA2A and then digested by FokI as described in Methods. Only a mutant allele produced 104 and 31 bp fragments (the smaller fragment is not shown in this figure) in addition to 135 bp PCR product. Closed symbols: clinically diagnosed VHL patients. Open symbols: clinically asymptomatic subjects. M: DNA size marker. (C) Detection of nt 686 T to C mutation. Four DNA samples from affected members in a VHL family 4475 (lanes 1-4), one from family 4408 (lane 5), and a normal control (lane 6) were amplified by PCR with primer TK3 and YH1A by *AccI* digestion. Mutant alleles created 68 and 30 bp fragments (only the larger fragment was shown) in addition to 98 bp PCR product. M: DNA size marker.

tests. On the other hand, the mutations found in the VHL type 1 families consisted of many types of large deletions, small deletions/insertions, or point mutations. No hot spots were found in the mutations of the VHL type 1 families,¹² so it is not practical to apply restriction enzyme based tests for screening of unknown mutations in the VHL type 1 patients.

Detection of mutations in VHL type 2 families by restriction enzyme

Mutation	Restriction site change	No of VHL type 2 families		
		USA ¹²	England ¹⁴	Germany ^{15*}
nt 712 C to T	MspI	4	3	0
nt 713 G to A	MspI	6	4	0
nt 505 T to C	FokI†	2	0	14
nt 686 T to C	AccI†	1	0	0
Total		13/23‡	7/11	14/14

* VHL families in the Black Forest area.

† Available by PCR with primer specified modification.

‡ Number of VHL type 2 families with nt 712, 713, 505, or 686 mutation/number of total VHL type 2 families.

Even with time consuming, expensive, and radioactive methods such as Southern blotting, pulsed field electrophoresis, and SSCP and DNA sequencing, mutations in the VHL gene could be detected in 70% of VHL type 1 families and 88% of VHL type 2 families.¹² Although much effort is still needed to detect mutations in the VHL type 1 patients, screening of mutations in the VHL type 2 patients can be simplified with restriction enzyme based tests. Our method proved to be useful not only in the USA but also in England and Germany (table).

In summary, we described a convenient method of detecting point mutations at nt 505 T to C and nt 686 T to C in the VHL gene and its possible role for the screening of subjects in VHL type 2 families.

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