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Short Report

Detection of a novel germline mutation in the von Hippel–Lindau tumour-suppressor gene by fluorescence-labelled base excision sequence scanning (F-BESS)

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The von Hippel–Lindau (VHL) syndrome is an inherited multi-tumour disorder characterised by clinical heterogeneity and high penetrance. The *VHL* gene has been shown to be a tumour-suppressor gene. A carrier of a germline mutation will be predisposed to a high variety of benign and malign tumours affecting different organ systems. As treatment of *VHL* malformations in presymptomatic stages will improve significantly the clinical outcome and the patient's quality of life, early and unambiguous detection of a germline mutation is mandatory. Direct sequencing especially of large genes might be laborious and time consuming. Therefore, most laboratories apply single strand conformational polymorphism (SSCP) analysis as an initial screening technique. Major disadvantages of this approach are the requirement of specialised equipment and a limited detection rate of about 70%. To overcome these problems, we applied the modified technique of fluorescence-labelled base excision sequence scanning (F-BESS). A young patient without family history of VHL with two hemangioblastoma of the cerebellum, pancreatic cysts and angiomatosis retinae was presented. Applying F-BESS, we detected a frameshift in exon 2 as a *de novo* germline mutation. Direct sequencing revealed an insertion of C at position 631/632. This is a novel VHL mutation, which results in truncation of the VHL protein omitting the Elongin-binding domain. Applying SSCP on the same DNA, no alteration could be detected. Three further family members were tested negative for the mutation by F-BESS in accordance with lack of any clinical *VHL* features. As F-BESS appears to be a reliable, fast and unexpensive method, we recommend this technique as an initial screening method.

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The von Hippel–Lindau (VHL) syndrome is an hereditary disease predisposing to multiple tumours or cysts in different organs, e.g. cerebellum, spinal cord, retina, adrenal gland, kidney and others. The disease is inherited in an autosomal dominant trait with a penetrance of about 90% at the age of 65 years. The majority of tumours are benign with the exception of renal cell carcinomas (RCC). The most severe clinical complications are metastatic spread of RCC, neurological problems from brain or spinal tumours and the loss of sight due to angiomatosis retinae. It is most beneficial to establish the diagnosis in a presymptomatic stage, as early detecion will allow prevention from most of these complications. Since the gene has been cloned (1), genetic testing is available (2, 3), leading to improved disease management. The VHL gene is encoded by 642 bp and consists of three exons. Mutations of the VHL gene have been shown to be the basis for the disease (4), including hypermethylation of 5′ CpG islands as a possible mechanism of *VHL* inactivation (5). Recent investigations showed that sporadic renal cell carcinomas of the clear cell subtype (6) are also

associated and very likely functionally related to deregulation of VHL protein (pVHL) via elongation ((7), for review see (8)). Therefore, fast and reliable genetic testing for VHL mutations is most important for the patients at risk, the clinicians and the scientists. Unnecessary and expensive diagnostic procedures for unaffected family members can be avoided.

No hot-spot mutation region in the gene has been found. Therefore, the method mostly used for genetic screening today is polymerase chain reaction (PCR)/single strand conformational polymorphism (SSCP) analysis of each exon followed by sequencing of the deviant DNA segment (9), or direct sequencing of the whole VHL gene after PCR amplification. In a comprehensive study summarising data on more than 460 VHL families, seven of the eight collaborating laboratories used PCR/SSCP (3). Only in 300 of the families tested, VHL mutations could be detected (63%). In some cases, family members can be analysed by restriction analysis, due to the generation or loss of a restriction site (10). Sequencing the whole gene is expensive and time consuming. Because of the known disadvantages of PCR/SSCP (11, 12), we investigated an alternative method.

The usage of DNA glycosylase (13) might be advantageous, because in 80–90% of all substitutions, a thymidine is involved at the sense or the antisense DNA strand, respectively. Replacing thymidine stochastically by uridinyl during PCR, and the subsequent digestion of these sites, a defined series of fragments can be generated for each sample. After comparison with a standard sample, mutations at specific sites can be detected. We adapted the previously described base pair excision sequence scanning (BESS) method (14) for fluorescence-labelled fragment analysis (F-BESS). Consequently, sense and antisense strand can be assessed in a single reaction. We compared SSCP with F-BESS and found F-BESS to be more sensitive in detecting this mutation. This innovation will hasten the analysis of VHL mutations and will be advantageous especially for screening large genes.

Patients, materials and methods

Patients

1) A 28-year-old woman presented with neurological symptoms from two haemangioblastomas (cerebellar and cerebro-spinal). After surgical removal of these tumours, the patient recovered without any problems. Refined clinical work-up revealed pancreatic cysts and an angiomatosis retinae. The early onset of haemangioblastomas and the additional clinical features classified the patient as a prime candidate suspicious of VHL disease. There were no other familiy members with any signs of VHL disease. The patient's father died from a myocardial infarction at the age of 58 years. DNA was available from her sister (32 years), brother (29 years) and mother (55 years).

2) To evaluate the sensitivity of the methods described, 22 sporadic clear cell renal carcinoma tumour samples were analysed.

Informed consent was obtained from all individuals tested in this study.

DNA isolation

DNA was extracted using the DNA Isolation Kit for Mammalian Blood (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. The dried DNA was resolved in H_2O and stored at $-20°C$ until further use.

PCR/SSCP analysis

SSCP analysis was performed as described earlier (9). In brief, exons one to three of the *VHL* gene were amplified by PCR. Aliquots of the PCR products were separated on horizontal nondenaturating 8% polyacrylamid gels containing 2% glycerol. Electrophoresis was performed using a TGGE system (Qiagen, Hilden, Germany). Subsequently, silverstaining was performed according to the manufacturer's protocol (Qiagen). As a normal control, DNA of a 22-year-old healthy person was included.

Fluorescence-labelled base excision sequence scanning (F-BESS)

We used the BESS T-Scan™ kit (Epicentre Technologies, Madison, WI). The major modification was replacement of ^{32}P-labelled primers by fluorescence-labelled primers. The sense primers were FAM- and the antisense primers HEX-labelled. All oligonucleotides used were obtained from MWG, Ebersberg, Germany.

Exon 1a Sense
5′FAM-AGC GCG TTC CAT CCT CTA CC-3′

Antisense
5′HEX-CGG CCT CCA TCT CCT CCT CG-3′

Exon 1b Sense
5′FAM-TGA AGA AGA CGG CGG GGA GG-3′

Antisense
5′HEX-TCA GAC CGT GCT ATC GTC CC-3′

Exon 2 Sense
5'FAM-TGT GGC TCT TTA ACA ACC TTT GC-3'

Antisense
5'HEX-TAT CCT GTA CTT ACC ACA ACA ACC-3'

Exon 3 Sense
5'FAM-CTA GTC TGT CAC TGA GGA TTT GG-3'

Antisense
5'HEX-CTG AGA TGA AAC AGT GTA AGT TTC-3'

The PCR mixture contained 1 U Taq (AmpliTaq Gold™, Perkin–Elmer, Weiterstadt, Germany), 10 × buffer (Gene Amp®, Perkin–Elmer, Weiterstadt, Germany), 1.5 mM $MgCl_2$, 0.4 µM primer (each), 200 µM dNTPs (each), 16 µM dUTP (BESS T-Scan dNTP Mix), 200 ng genomic DNA and H_2O in a finale volume of 25 µl. The amplification was performed as follows: an initial denaturation step (5 min, 94°C) was followed by 40 cycles. Each cycle was performed with a 1-min denaturation at 94°C, annealing at 55°C for 1 min and extension for 2 min at 72°C. The reaction was finished by adding an elongation reaction for 20 min at 72°C. The mixture was cleaned by gel elution (GFX-Kit, Pharmacia, Uppsala, Sweden). During amplification, dUTP and dTTP were incorporated into the DNA product. Subsequently, the excision/cleavage reaction was performed. For this purpose, the eluent (8 µl) of the labelling amplification reaction was mixed with 1 µl T-Scan buffer, 0.5 µl enzyme mix and 0.5 µl H_2O, incubated at 37°C for 40 min and stopped by the addition of 5 µl Stop/loading buffer. Three microlitres of the reaction were mixed with 0.4 Standard (Genescan™-350, TAMRA, Perkin–Elmer, Weiterstadt, Germany) for estimation of the fragment lengths, and after denaturation (5 min, 75°C) subjected to electrophoresis on a 7-M urea, 4.25% polyacrylamid sequencing gel. Separation of the fragments was performed for 4.5 h and 2400 scans/h using an ABI prism™ 377XL-sequencer.

Uracil N-glycosylase (UNG) removes uracil bases from the nucleotides. Endonuclease IV, as part of the enzyme mixture, cleaves the phosphodiester bonds at these abasic sites, creating a defined series of fragments. Eletrophoretic separation will resemble and correspond to the 'T lane' of a conventional sequencing reaction. An alteration of the banding pattern (appearance, disappearance or change in intensity compared to the lane with the wild type reaction) will indicate a mutational event. Applying this technique, simultaneously using differently labelled 5 and 3 primers, will confirm the detection and location of a mutation involving thymidine.

Direct sequencing

The same DNA samples used for SSCP analysis were reamplified using internal oligonucleotides in an independent reaction. Analysis was performed on an automated sequencer (LiCor 4200 iR2, MWG, Ebersberg, Germany) according to the manufacturer's instructions.

Restriction analysis

An aliquot (200 ng) of each DNA sample obtained from four family members was analysed by restriction digestion. The restriction was performed at 37°C for 2 h using 5 U MnlI (MBI Fermentas, St. Leon-Rot, Germany). The fragments were compared by electrophoresis on a 2% agarose gel followed by ethidium bromide staining.

Results

Using PCR/SSCP, F-BESS, restriction analysis and direct sequencing, we searched for *VHL* germline mutations in four members of a family. In this pedegree, only one person has been at risk for the disease. Applying PCR/SSCP on all three *VHL* exons, no deviant DNA band was detectable in any of the 4 patients' samples (data not shown). However, using F-BESS, we have been able to detect a heterozygous mutation in the only patient at risk: additional fragments starting at position 631 indicated an insertion of a nucleotide at this position in exon 2. Nucleotides were numbered according to the cDNA sequence published by Latif et al. (1). Due to this frameshift, a 'shadow sequence' accompanying the wild type sequence was seen (Fig. 1). The remaining family members showed a *VHL* wild type pattern for all exons. The F-BESS data were confirmed by direct sequencing of all samples. The sequence analysis revealed an insertion of a cytosin at position 631 (Fig. 2), resulting in a frameshift that is in accordance with the F-BESS result. This insertion is a novel germline mutation that has not been described before (3, 15). It generates a new MnlI restriction site. We digested exon 2 amplificats of all family members with the restriction endonuclease MnlI. Again, the mutational status of all family members was confirmed (Fig. 3).

To analyse the reliability and sensitivity of F-BESS, we analysed 22 RCC samples (Table 1) by SSCP, F-BESS and direct sequencing. Prior to F-BESS analysis, 16 out of these 22 samples were classified as SSCP-positive, six as SSCP-negative. Seven out of these 16 SSCP-positive samples were also F-BESS-positive. Subsequent sequencing of all samples confirmed mutations of all seven samples. No false positive samples resulted from F-BESS, whereas 8 out of 16 SSCP-positive samples did not show any mutation by sequencing. One C→G mutation (ST56) was not detected by F-BESS due to limitations inherent to this method. One sample

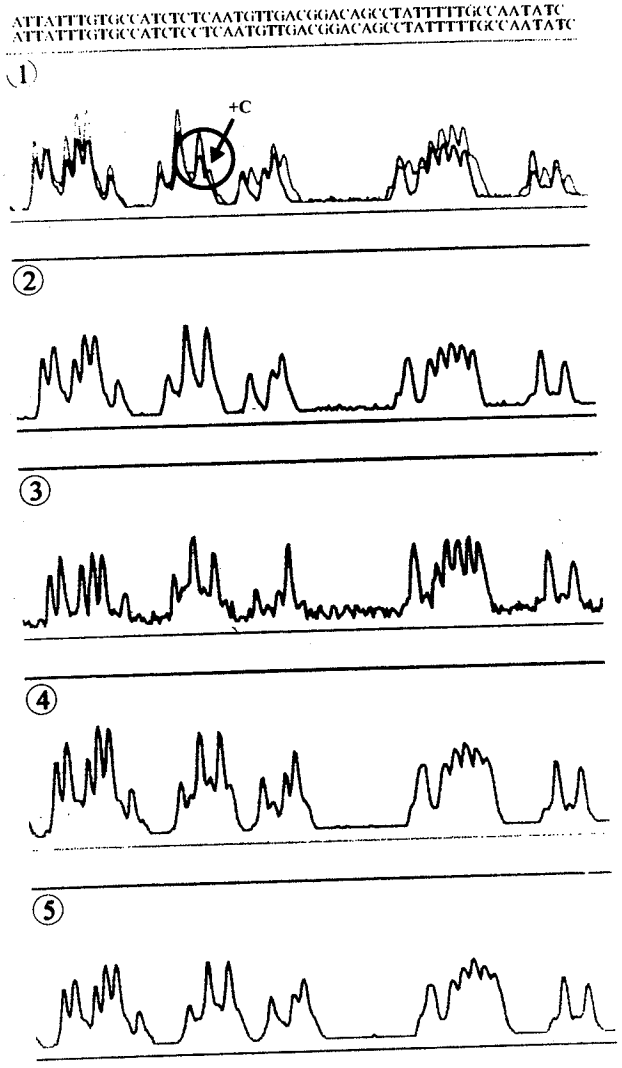

Fig. 1. F-BESS analysis of amplified DNA: DNA was isolated and amplified using exon specific primers. The 5′-BESS analysis for exon 2 harbouring the mutation is shown. A bandshift of one allele due to an additional C (arrow) was detected. For comparison purposes a wild type sample from a healthy person was processed in parallel. For length estimation, the ABI Standard TAMRA-350 was included in each sample before electrophoresis (not shown). 1: Patient (light) and healthy volunteer (dark); 2: control; 3: mother; 4: brother; 5: sister.

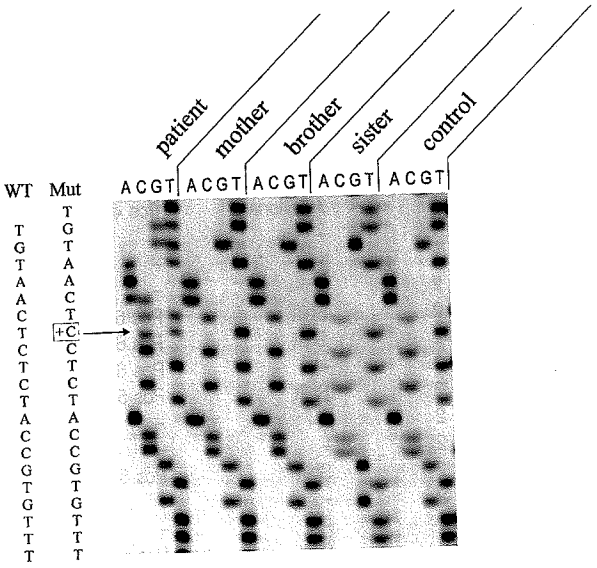

Fig. 2. Sequence analysis of exon 2: Insertion of a C, causing the BESS bandshift shown in Fig. 1, can easily be recognised (arrow).

was SSCP-negative (ST42), whereas F-BESS on this specimen was indicative of a mutation, which was confirmed by direct sequencing.

Discussion

Clinical management of *VHL* will be greatly improved by unambiguous diagnosis applying genetic testing in presymptomatic stages. This applies for many of the hereditary tumour-predisposing syndromes. Appropriate medical and psychological precautions can be taken, depending on knowledge of the unequivocal mutation status.

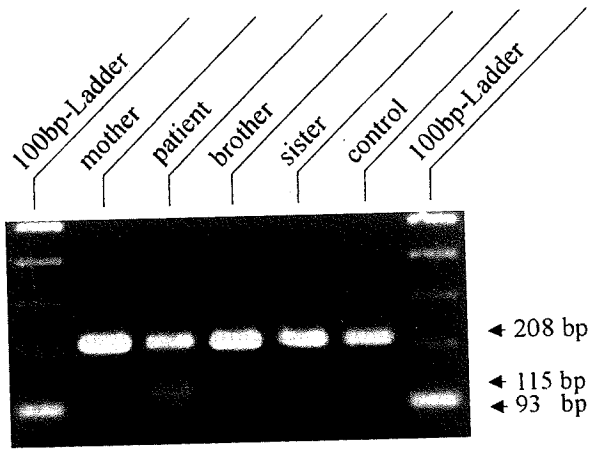

Fig. 3. Detection of the C insertion at nt 631 by restriction analysis: PCR product of exon 2 (208 bp) was subjected to digestion by the restriction endonuclease MnlI. The mutant allele of the patient has been cut showing two fragments of 115 and 93 bp.

Table 1. Analysis of 22 sporadic RCC for VHL mutations

Patient no/sporadic RCC	Exon no.	F-BESS	Sequencing
Aberrant band pattern detected by SSCP			
ST10	1	–	–
ST52	1	Additional T	Pos 416 C→A
ST84	1	–	–
ST202	1	–	–
ST14	2	–	–
ST22	2	Shift	Pos 663 T→A
ST36	2	Shift	Pos 636 ins A
ST56	2	–	Pos 642 C→G
ST72	2	Shift	Pos 563 del G
ST82	2	–	–
ST96	2	–	–
ST114	2	Shift	Pos 591 del T
ST130	2	–	–
ST160	2	–	–
ST168	2	Shift	Pos 558 del C
ST48	3	Reduced T-signal	Pos 686 T→C
No aberrant band pattern detected by SSCP			
ST20	–	–	–
ST40	–	–	–
ST42	–	Additional T	Pos 287 C→T
ST92	–	–	–
ST134	–	–	–
ST148	–	–	–
VHL family			
Patient	–	Shift	Pos 631 ins C
Mother	–	–	–
Brother	–	–	–
Sister	–	–	–

No false positive samples resulted from F-BESS compared to eight false positive samples detected by SSCP. One mutation (ST56, C→G) was not detectable by F-BESS due to its methological limitation. In one sample (out of six) without SSCP detection, a point mutation was detected by F-BESS (–, no mutation detected). The described VHL family is depicted for entirety.

Detecting a *VHL* mutation in a person at risk can be a problem for families where the mutation has not yet been established. As a screening method, PCR/SSCP is one of the techniques most widely used. Though some groups report on a sensitivity of this technique of up to 90% (12), there are substantial discrepancies in the reported sensitivity of the PCR/SSCP approach (11). In a recent comprehensive study summarising the germline mutations of 469 *VHL* families, seven (out of eight) laboratories used the PCR/SSCP approach for mutation screening. Comparing the success rate of these laboratories, the sensitivity in larger cohorts varied substantially, e.g. from 33% (18/54) to up to 76% (37/49). To our experience, slight variation of the gel, the electrophoretic conditions or the purity of the sample can easily interfere with the result. It might be realistic to assume that a considerable number of mutations will be missed using the PCR/SSCP approach. Therefore, we looked for an alternative method as a mutation screening approach in *VHL*.

We modified the BESS method by replacing ^{32}P-labelled primers by fluorescence-labelled primers. This method omits the extensive optimisation of SSCP conditions, and no special gels or specialised equipment are required. The BESS technique allows detection of all frameshifts, insertions, deletions and of 80–90% of all point mutations. The disadvantage is that G→C and C→G point mutations are not detectable, which might account for about 10% of all VHL mutations (3). Advantages of BESS are the larger fragments (500–600 bp) that can be analysed compared to about 200 bp in SSCP, and the gain of initial information on the location of the mutation thus speeding up the subsequent sequence analysis. We applied the F-BESS technique for germline mutation analysis on four members of a family including a young female patient at risk for VHL. A mutation (Fig. 1) could be detected, which had been missed using the PCR/SSCP approach. We confirmed a C insertion at position 631 by direct sequencing.

The *VHL* gene encodes a protein that regulates the transcription elongation by binding to elongin B and C. The COOH terminus harbours the elongin-binding site from AS 157–189 (16–20). The novel mutation we detected at position 631 (AS 139) will result in a truncated VHL protein, missing the elongin-binding domain. Therefore, the mutant VHL protein will fail to interact with the elongin B/C complex. Consequently, a transcriptional dysregulation of target genes will occur that ultimately might lead to neoplasia. Applying a thorough clinical work-up, no pheochromocytoma was found in our patient. This is in line with recently published studies showing that *VHL* patients without pheochromocytomas rarely harbour deletions, insertions or nonsense mutations (2, 3).

Besides our index patient, none of the family members showed typical VHL symptoms. This is consistent with the *VHL* wild type status of the three other persons we tested. Therefore, the mutation appears to be a *de novo* mutation.

In addition, we used the restriction analysis technique to confirm presence or absence of the C insertion detected (Fig. 3). If technically possible, this method is extremely helpful in speeding up the identification of the mutational status for a person in a VHL pedigree. Prerequisite is that a restriction site will be generated or omitted by the mutation.

In sporadic renal clear cell carcinoma (ccRCC), the *VHL* gene is affected in up to 80% (21, 22). As no mutation hot spots have been found for ccRCC, a high throughput mutation screening technique will be greatly appreciated. We therefore

analysed 22 sporadic renal cell carcinoma by SSCP, F-BESS and direct sequencing. In this context, we found F-BESS to be a fast, reliable and advantageous method compared to the SSCP.

In our study, we showed that the modified F-BESS technique can be applied successfully for VHL mutational screening. In our hands, F-BESS represents an alternative method to SSCP. Further studies in different laboratories targeting different genes should be performed to show the reproducibility and the advantages of this technique. Therefore, F-BESS should increasingly be considered as an alternative technique for initial mutation screening. Genetic analysis of larger genes in other hereditary syndromes and mutational screening in sporadic tumours will benefit from this new technique.

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