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ORIGINAL INVESTIGATION

Bettina Klein · Gregor Weirich · Hiltrud Brauch

DHPLC-based germline mutation screening in the analysis of the *VHL* tumor suppressor gene: usefulness and limitations

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Abstract In order to evaluate the sensitivity and specificity of the recently introduced high-throughput method DHPLC (denaturing high performance liquid chromatography) for mutation screening in the *VHL* tumor suppressor gene, we subjected DNA from 43 unrelated VHL patients with previously sequenced *VHL* germline mutations to this method. In addition, 36 genomic DNAs of unrelated individuals suspected of being *VHL* carriers but with unknown germline status were analyzed by DHPLC and sequencing. The aims of the present study were to compare mutation results obtained by direct sequencing and DHPLC, and a comparison of two different DHPLC systems. The sensitivity of DHPLC was tested with two commercial devices and protocols, i.e., the Varian-Helix system and the Wave Nucleic Acid Fragment Analysis system. Both resolved all but one mutation in exons 2 and 3 of the *VHL* gene. In contrast, the GC-rich exon 1 showed discrepancies in the rate of mutation detection. Whereas the Varian-Helix system detected 10/15 (67%) of the known mutations, the Wave Nucleic Acid Fragment Analysis system detected 13/14 (93%). All three mutations in samples with unknown mutation status were revealed by both systems raising the mutation detection rate to 72% and 94%, respectively. Cases with different substitutions at the same nucleotide showed different elution profiles, but similar elution profiles could be obtained from different mutations. The Wave Nucleic Acid Fragment Analysis system detected most *VHL* mutations; however, when a 100% detection rate is needed, sequencing is still required and must therefore be the standard *VHL* mutation detection procedure. Once a family-specific mutation has been established, DHPLC may be suitable for the rapid and cost-effective determination of *VHL* carrier status in family members.

B. Klein · H. Brauch (✉)
Dr. Margarete Fischer-Bosch-Institut für Klinische Pharmakologie,
Auerbachstrasse 112, 70376 Stuttgart, Germany
e-mail: hiltrud.brauch@ikp-stuttgart.de,
Tel.: +49-711-81013705, Fax: +49-711-859295

G. Weirich
Institut für Pathologie, Technische Universität München,
Trogerstrasse 18, 81675 München, Germany

Introduction

Germline mutations in the *VHL* tumor suppressor gene predispose carriers to develop von Hippel-Lindau (VHL) disease, a multitumor syndrome that includes tumors in eyes, brain, spinal cord, kidney, pancreas, adrenal gland, epididymis, and inner ear (Glenn et al. 1990; Manski et al. 1997; Neumann 1987). The *VHL* gene was identified in 1993 (Latif et al. 1993), and since then, more than 500 VHL families world-wide have enroled in germline mutation testing (Beroud et al. 1998, 2000; Zbar et al. 1996). Individuals at risk for VHL, i.e., members of families with a known family history, and isolated cases with an incomplete phenotype should benefit from molecular testing with respect to: (1) confirmation of clinical diagnosis, (2) presymptomatic diagnosis, and (3) exclusion from being a carrier of a family-specific *VHL* mutation. Although at present there is no cure, molecular diagnosis of VHL disease has had a major impact on clinical VHL management resulting in the prevention of blindness, neurological impairment, and metastatic disease in affected patients (Neumann et al. 1995). This improvement in patient care and prevention relies on the early determination of gene carrier status and subsequent regular organ-specific clinical screening for lesions in *VHL* gene carriers. Thus, good clinical practise in the mangement of VHL disease must include germline mutation analysis.

Reliable and sensitive methods for the detection of DNA sequence variations are pivotal for the identification of disease-causing germline mutations. Modern DNA technology has provided a number of semi-automated DNA screening tools, such as denaturing gradient gel electrophoresis and single-strand conformation polymorphism analysis (SSCP), which, however, have shortcomings regarding their accuracy, practicability, and costs. Whereas direct sequencing is most accurate, it still presents a financial burden in high-throughput facilities. For this rea-

son, fast and reliable DNA screening procedures are in demand. Denaturing high performance liquid chromatography (DHPLC) is a recently introduced high-throughput method that facilitates mutation detection of short double-stranded DNA molecules (Oefner and Underhill 1998). This method is based on mismatched heteroduplex formation between wild-type and mutant DNA single strands. These mismatched heteroduplexes and matched wild-type and mutant homoduplexes differ from each other in their thermostability. When separated by means of a chromatographic column at a sequence-specific melting temperature and gradient elution buffer (acetonitrile), heteroduplexes and homoduplexes will elute at different times, giving rise to complex chromatograms. Therefore, the presence of a one-peaked chromatogram is evidence of wild-type DNA, whereas complex chromatograms (two or more peaks) indicate the presence of wild-type and mutant DNA. When applied to germline mutation screening of the *BRCA 1* and *BRCA 2* gene in patients at risk for hereditary breast cancer or of the *MET* proto-oncogene in patients at risk for familial papillary renal cell carcinoma, sequence variation detection by DHPLC was 100% accurate (Arnold et al. 1999; Gross et al. 1999; Nickerson et al. 2000). DHPLC was also superior to SSCP analysis in *BRCA 1* (Gross et al. 1999), *TSC 1* and *TSC 2*, and in non-cancer genes, such as *CFTR* (Jones et al. 1999; Jones et al. 2000). In order to test the applicability of the DHPLC method for the detection of *VHL* germline mutations, we subjected over 40 different, previously established *VHL* germline mutations (Glavač et al. 1996; H. Brauch, unpublished) to DHPLC analysis. In addition, we compared the results of DHPLC and sequencing analyses of patient samples with unknown *VHL* mutation status. We herein provide an overview of the usefulness and limitations of the DHPLC method in *VHL* germline mutation screening.

Table 1 DNA samples analyzed in DHPLC-assisted *VHL* mutation analysis

Patient details	Number
VHL patients with confirmed *VHL* mutation (VHL 1–104)	43
Missense mutation	21
Nonsense mutation	10
Splice site mutation	5
Frameshift mutation	4
In frame insertion	1
In frame deletion	1
3'UTR mutation	1
Patients at risk for *VHL* mutation	36
Patients with clinically diagnosed VHL disease	5
Patients suspected as having VHL disease	20
Patients with pheochromocytomas	5
Patients with kidney cancer	6

Table 2 *VHL* germline mutations analyzed and detected by DHPLC. Families in *bold* represent families whose mutations were not included in Glavač et al. (1996). Exonic nucleotides are *capitalized*, and intronic nucleotides are in *lower case* (*ins* insertion, *del* deletion, *n.a.* not analyzed). Alterations in exonic sequences are indicated either by the exact nucleotides or the 5'-most nucleotide. Likewise, codons are numbered when directly affected or by the last intact codon in the case of deletion or insertion mutations. VHL 57 has been included to allow comparison with previous analyses, although it was not available and therefore not analyzed in this study

Exon	Family [no.]	Nucleotide change	Protein change	DHPLC Varian-Helix temperature	DHPLC Wave temperature
Exon 1 (28/22)				63°C	69°C
	VHL [**73**]	396 insC	Pro61, truncation	+(new)	+
	VHL [**83**]	407 C→A	Ser65Stop	+	n.a.
	VHL [50]	421 G→T	Glu70Stop	–	+
	VHL [9, **92**, 53]	434 T→G	Val74Gly	+	+
	VHL [26, **63**, **77**]	440 delTCT	Ile75, truncation	+	+
	VHL [51]	446 A→G	Asn78Ser	+	+
	VHL [39, **82**]	452 G→A	Ser80Asn	+	+
	VHL [**104**]	452 G→T	Ser80Ile	+	+
	VHL [23, 38]	454 C→T	Pro81Ser	–	–
	VHL [110]	463 G→T	Val84Pro	+(new)	+
	VHL [**66**]	467 T→C	Leu85Pro	–	+
	VHL [3]	479 T→C	Leu89Pro	–	+
	VHL [62][a]	490 G→A	Gly93Ser	–	+
	VHL [106]	490 G→C	Gly93Arg	+(new)	+
	VHL [**85**]	493 G→T	Glu94Stop	+	+
	VHL [1, founder mutation[b]]	505 T→C	Tyr98His	+	+
	VHL [**86**]	537 insCGC	Arg108, insArg	+	+
	VHL [55]	550 C→T	Arg113Stop	+	+
Rate of detection	Previously known			10/15 (67%)	13/14 (93%)
	All			3/18 (72%)	16/17 (94%)

Table 2 (continued)

Exon	Family [no.]	Nucleotide change	Protein change	DHPLC Varian-Helix temperature	DHPLC Wave temperature
Exon 2 (I5/I3)				59°C	60°C
	VHL [59]	556 C→T	His115Tyr	+	+
	VHL [60]	557 A→G	His115Arg	+	+
	VHL [**94**]	566 T→C	Leu118Pro	+	+
	VHL [**99**]	570 C→G	Phe119Leu	+	+
	VHL [**105**]	575 A→G	Asp121Gly	+	n. a.
	VHL [44]	597 delT	Leu127, truncation	+	+
	VHL [30]	607 C→T	Gln132Stop	+	+
	VHL [**78**]	620 T→G	Phe136Cys	–	–
	VHL [**75**]	662 del14nt	Ala149, truncation	+	+
	VHL [45]	676+1 g→c	Splice defect	+	+
	VHL [7][a]	676+2 t→c	Splice defect	+	+
	VHL [**97**, **102**]	676+8 c→t	Splice defect	+	+
Rate of detection:				11/12 (92%)	10/11 (91%)
Exon 3 (YH1A/6b)				60°C/62°C	60°C/62°C
	VHL [57]	677-2 a→t	Splice defect	n.a.	n.a.
	VHL [31]	677-2 a→g	Splice defect	+	+
	VHL [48]	677-1 g→a	Splice defect	+	+
	VHL [49, 32]	694 C→T	Arg161Stop	+	+
	VHL [13]	694 C→G	Arg161Gly	+	+
	VHL [42]	699 C→G	Cys162Trp	+	n.a.
	VHL [**90**]	699 insG	Arg161, truncation	+	n.a.
	VHL [65]	703 C→T	Glu164Stop	+	n.a.
	VHL [**81**, **84**, **95**, **96**, **109**]	712 C→T	Arg167Trp	+[c]	+[c]
	VHL [43, **76**, **107**]	713 G→A	Arg167Gln	+[c]	+[c]
	VHL [**103**]	738 C→G	Tyr175Stop	+	+
	VHL [29]	746 T→A	Leu178Gln	+[c]	+[c]
	VHL [6]	761 C→A	Ser183Stop	+[c]	+[c]
	VHL [37, 38]	775 C→G	Leu188Val	+[c]	+[c]
	VHL [**79**]	794/795 delTG	Asp194, truncation	+	+
	VHL [56]	796 C→T	Gln195Stop	+[c]	+[c]
	VHL [**80**]	852+10 c→g	3'UTR	+	+
Rate of detection:				16/16 (100%)	13/13 (100%)

[a]Mutations at nucleotides corrected compared with Glavac et al. (1996)
[b]Families with a founder mutation were VHL 1, 2, 4, 5, 8, 11, 12, 16, 17, 18, 22, 27, 28, 34, 47, 54, 3127, and 3476 (Brauch et al. 1995)
[c]Samples presented at 60°C with a wild-type elution profile or with a peak shoulder but presented at 62°C with two peaks

Materials and methods

Patients

Genomic DNA of 43 unrelated VHL patients with different previously established *VHL* germline mutations were subjected to the polymerase chain reaction (PCR) and DHPLC (Table 1; Glavač et al. 1996; Zbar et al. 1996). Samples encoded VHL 1–65 (Table 2) represented patients of VHL families, whose *VHL* germline mutations had been published previously (Glavač et al. 1996), and samples encoded VHL 66–110 (Table 2) represented patients of families whose family-specific *VHL* mutations had been identified in our laboratory from 1996 to the present.

VHL mutations included nucleotide and splice site variations extending from exon 1 (nucleotide 407) to exon 3, and the 3' untranslated region (3'UTR: nucleotide 852+10). There were 21 missense, 10 nonsense, four frameshift mutations, one inframe insertion, one inframe deletion, and five splice site mutations, and one nucleotide change at the 3'UTR. Fifteen mutations were located within exon 1, 9 mutations within exon 2, and 13 mutations within exon 3. Three mutations were located at the splice donor and two mutations at the splice acceptor site of intron 2. One mutation was located in the 3'UTR.

In addition, 36 genomic DNAs of unrelated individuals with unknown *VHL* germline status were tested (Table 1). These patients were referred to us from various physicians and human genetics departments in Europe for *VHL* mutation analysis in order to assist clinical diagnosis. Patients were considered to be at various degrees of risk of being *VHL* mutation carriers. Samples included DNAs from five clinically diagnosed VHL patients who were not detected as having any *VHL* germline mutation when screened by SSCP analysis, 20 patients suspected as having VHL disease not further specified, five patients with pheochromocytoma (one fa-

milial and four diagnosed below 50 years of age), and six patients with kidney cancer (five with family history and one associated with cysts). We also included non-symptomatic offspring of the patient with familial pheochromocytoma in the analyses (two subjects).

Controls

Five genomic DNAs of known *VHL* wild-type homozygous individuals were used to establish DHPLC wild-type elution profiles for each *VHL* exon.

DNA isolation and previous mutation detection procedures

Genomic DNA of all individuals was isolated from whole blood samples according to standard phenol/chloroform extraction procedures (Sambrook et al. 1989). Known mutations to be analyzed by DHPLC in this work had been previously identified by SSCP screening and sequencing of samples with aberrant SSCP pattern (Glavač et al. 1996; Zbar et al. 1996).

PCR and DHPLC analysis

PCR of the *VHL* gene was performed with three primer sets, viz., VHL 28 and VHL 22, I5 and I3, and YH1A and 6b, according to published procedures (Glavač et al. 1996). Modification included the use of the Advantage-GC cDNA Kit, including a mix of Klen Taq-1 DNA polymerase with a minor amount of a 3'>5'proofreading polymerase and TaqStart antibody (Clontech, Palo Alto, Calif., USA) for amplification of exon 1 (VHL28/VHL22) with a final GC-Melt concentration of 1.0 M. PCR of exon 2 (I5/I3) and exon 3 (YH1A/6b) did not contain additives, and the *Taq* polymerase was AmplitaqGold (Perkin Elmer, Branchbury, N.J., USA). Reactions were carried out in a 96-well plate sealed with Eppendorf heat sealing foil (Eppendorf Scientific, Westbury, N.Y., USA). The reaction volume was 25 µl, and thermocycling was for 35 cycles in a PTC 225 Peltier Thermal Cycler (MJ Research, Waltham, Mass., USA). All PCR products were checked for specificity on a 2% agarose gel prior to DHPLC. Exon 1 PCR products were gel-purified in 1.9% low-melting agarose (1.5% NuSieve GTG agarose and 0.4% Seakem ME agarose; BioWhittaker Molecular Applications, Rockland, Me., USA) to remove any PCR additives that were included in the kit and that may influence the column run, followed by extraction with the QIA quick Gel Extraction Kit (Qiagen, Hilden, Germany).

DHPLC analysis was performed according to Oefner and Underhill (1998). We compared the sensitivity of *VHL* mutation detection of two commercial devices, the Varian-Helix system (Varian, Walnut Creek, Calif., USA) and the Wave Nucleic Acid Fragment Analysis system HSM (Transgenomic, Omaha, Neb., USA).

Prior to DHPLC analysis, PCR products were denatured at 95°C for 5 min and gradually cooled down to 65°C at 1°C/min decrements. DHPLC was run under precise temperature control to seperate double-stranded DNA amplicons according to the degree of their single-stranded character. The column temperature was adjusted according to the program-specific calculated melting temperature of the double-stranded DNA amplicons to be analyzed. Optimal DNA melting temperatures for specific PCR products were calculated with the DHPLC Melt program available at the Stanford DHPLC web site, viz., http://insertion.stanford.edu/melt.html (Jones et al. 1999) and the WAVEMaker software of the Wave Nucleic Acid Fragment Analysis system HSM device. Both systems were exclusively used with their specific hardware and software. For *VHL* exon 1, the Stanford DHPLC Melt program calculated melting temperatures of 63–65°C, the WAVEMaker software of 68–70°C. For *VHL* exon 2, calculated melting temperatures were 55–59°C and 59–60°C, respectively; for exon 3, 55–60°C and 60–62°C, respectively. DHPLC analyses were performed within the calculated temperature ranges at 1°C increments.

Varian-Helix system

Samples of 5 µl were automatically injected onto an Eclipse dsDNA preparative 4.6×75 mm reversed phase column (Hewlett Packard, Palo Alto, Calif., USA). DNA was eluted within a total elution time of 8.5 min in a linear acetonitrile gradient with buffer A, i.e., 0.1 M triethylammonium acetate (TEAA), 0.1 mM EDTA, and with buffer B, i.e., 0.1 M TEAA, 25% acetonitrile, 0.1 mM EDTA. During a period of 7 min, the gradient increased from 40% to 95% buffer B. At peak elution, the content of buffer B was 70%–75%. Within the final 1.5 min, buffer B decreased to 40%. The buffer flow rate was constant at 1 ml/min. For the detection of *VHL* germline mutations, elution profiles of samples suspected of containing a mutation were compared with elution profiles of wild-type sequences for each exon. An evaluation of variations in elution profiles attributable to sequence changes was performed with Star Chromatography Workstation, Version 5 (Varian, Walnut Creek, Calif., USA).

Wave Nucleic Acid Fragment Analysis system

Samples of 5 µl were injected onto the DNA Sep Column (Transgenomic, Omaha, Neb., USA). The buffers used were of same TEAA concentration and acetonitrile content as those employed with the Varian-Helix system. The total run-time was 7.7 min. The gradient started with 48% buffer B increasing to 100%. Peak elution was at 60%–65% buffer B. The flow rate was constant at 0.9 ml/min. An evaluation of elution profiles was performed with the Wave software provided by the manufacturer.

DHPLC analyses of samples with known *VHL* mutations were carried out for each exon in a blinded fashion. Five wild-type samples and two positive controls were included in the test series. In order to be able to detect mutation-associated variations between DHPLC runs, three wild-type samples were run before and after each test series. In the test series, the order of mutant, wild-type, and positive control samples was unknown to the person carrying out the experiment. Deviations from the wild-type elution profile were noted by visual inspection when wild-type samples before and after the run were superimposed, indicating the stability of the DHPLC runs.

Sequencing

All 36 DNA samples of unknown *VHL* germline status were analyzed by DHPLC and sequencing at all three *VHL* exons. DNA was amplified in 100-µl volumes, purified, and sequenced by the dye terminator method on a automated 373 DNA sequencing device as described previously (Glavač et al. 1996).

Results

DHPLC mutation detection of known *VHL* germline mutations

We tested the sensitivity and efficiency of DHPLC analysis as a mutation screening method for the detection of 43 different *VHL* germline mutations. Scores obtained from the two commercial DHPLC analytical systems are given in Table 2 for melting temperatures giving the best resolution.

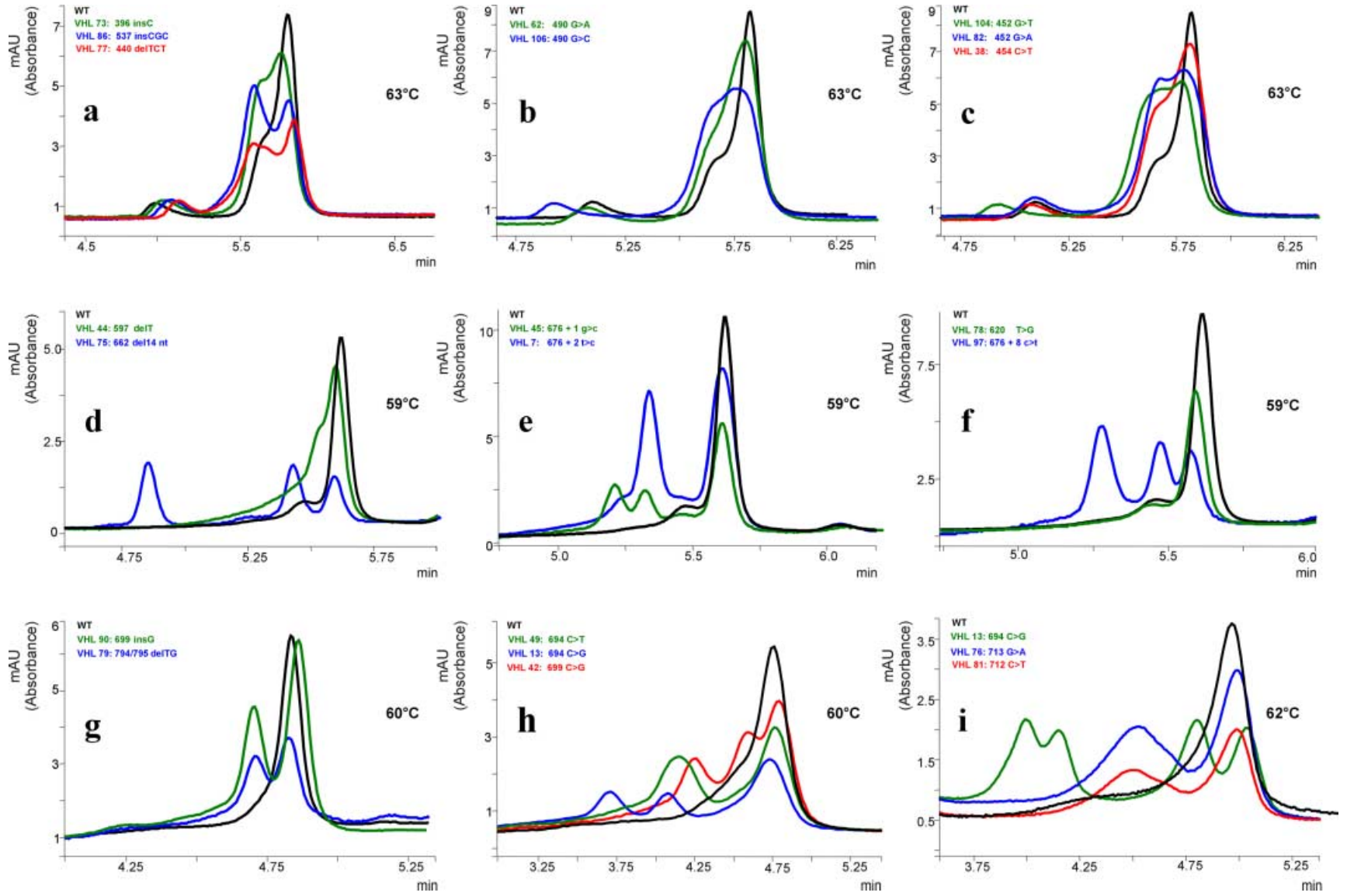

Fig. 1 Elution profiles of *VHL* mutations in exon 1 (**a–c**), exon 2 (**d–f**), and exon 3 (**g–i**) obtained with the Varian-Helix system. Insertions and deletions are presented in **a**, **d**, and **g**. Single nucleotide changes are presented in **b**, **c**, **e**, **f**, **h**, and **i**. VHL family mutations at the same nucleotide position, i.e., VHL 62: 490 G→A and VHL 106: 490 G→C in **b** show different elution profiles, with VHL 106: 490 G→A not being distinguishable from the wild-type. Likewise, different mutations for VHL 49: 694 C→T and VHL 13: 694 C→G in **h** show different elution profiles, whereas differences for VHL 104: 452 G→T and VHL 82: 452 G→A are not unanimously distinguishable (**c**). VHL 13: 694 C→G is an example of a mutation profile with three peaks at 60°C (**h**) and four peaks at 62°C (**i**). Mutations at VHL 76: 713 G→A and VHL 81: 712 C→T show identical elution profiles at 62°C (**i**). Mutations VHL 38: 454 C→T and VHL 78: 620 T→G did not show variant elution profiles when compared with the corresponding wild-type elution profile (**c, f**)

The Varian-Helix system detected 10/15 (67%) of the *VHL* mutations in exon 1, 11/12 (92%) mutations in exon 2 including the splice donor site of intron 2, and 16/16 (100%) mutations in exon 3 including the splice acceptor site of intron 2 and the 3'UTR (Table 2). All known deletions (n=4) and insertions (n=2), and 31/37 (84%) point mutations were detected. In all, the *VHL* mutation detection rate was 37/43 (86%).

When compared with mutation detection in exons 2 and 3, mutations in exon 1 were not as easily detected, with 33% escaping detection. Examples of elution profiles of *VHL* exon 1 mutations are given in Fig. 1a–c. Unanimous elution profiles were obtained from amplicons with deletions and insertions of more than one nucleotide, as shown for VHL 86: 537 insCGC and VHL 77: 440 delTCT (Fig. 1a). In contrast, single nucleotide insertion and changes presented with a broadening of the wild-type peak or poorly resolved double peaks. Examples are VHL 73: 396 insC (Fig. 1a), VHL 106: 490 G→C (Fig. 1b), and VHL 82: 452 G→A and VHL 104: 452 G→T (Fig. 1c). Mutations not detected in exon 1 are VHL 62: 490 G→A (Fig. 1b), VHL 38: 454 C→T (Fig. 1c), VHL 50: 421 G→T, VHL 23: 454 C→T, VHL 66: 467 T→C and VHL 3: 479 T→C (not shown).

All but one *VHL* mutation in exons 2 and 3 were detectable, with examples given in Fig. 1d–i. The one mutation that was not detectable was VHL 78: 620 →>G (Fig. 1f). Different mutations at the same nucleotide position showed different elution profiles as seen for VHL 49: 694 C→T and VHL 13: 694 C→G (Fig. 1h). A comparison of VHL 42: 699 C→G and VHL 13: 694 C→G shows that, in addition to peak number, the retention time and peak shape are important characteristics of mutant elution profiles. Resolution of VHL 13: 694 C→G changed from three peaks at 60°C (Fig. 1h) to four peaks at 62°C (Fig. 1i). Likewise, the resolution of the following mutations in exon 3 improved on changing the melting temperature from 60°C to 62°C: VHL 81: 712 C→T, VHL 76: 713 G→A, VHL 29: 746 T→A, VHL 6: 761 C→A, VHL 37 and 38: both 775 C→G, and VHL 56: 796 C→T (not shown).

Not all different mutations showed specific elution profiles. Neighboring mutations of VHL 81: 712 C→T and VHL 76: 713 G→A showed identical elution profiles at 62°C (Fig. 1i) and were not distinguishable from wild-type at 60°C (not shown). Identical elution profiles were also obtained for the insertion and deletion mutations at different nucleotide positions. Examples are VHL 90: 699 insG and VHL 79: 794/795 delTG (Fig. 1g).

The Wave Nucleic Acid Fragment Analysis system detected 13/14 (93%) of the *VHL* mutations in exon 1, 10/11 (91%) of the *VHL* mutations in exon 2 including the splice donor site of intron 2, and 13/13 (100%) mutations in exon 3 including the splice acceptor site of intron 2 and the 3'UTR. The combined *VHL* mutation detection rate was 36/38 (95%). The two false negative results obtained with both systems were the 454 C→T change in exon 1 and the 620 T→G change in exon 2 of the VHL families VHL 23 and 38, and of family VHL 78, respectively. We observed that three different mutations may present with similar elution profiles, as shown for examples VHL 50: 421 G→T and VHL 110: 463 G→T (Fig. 2a) and VHL 104: 452 G→T (Fig. 2c). We also observed that, in contrast to the DHPLC analysis with the Varian-Helix system, different mutations at the same nucleotide position, such as in VHL 38 and 82: 452 G→A and in VHL 104: 452 G→T may show different elution profiles with the Wave Nucleic Acid Fragment Analysis system (Figs. 1c, 2c). The overall sensitivity of the Wave Nucleic Acid Fragment Analysis system was superior to that of the Varian-Helix system.

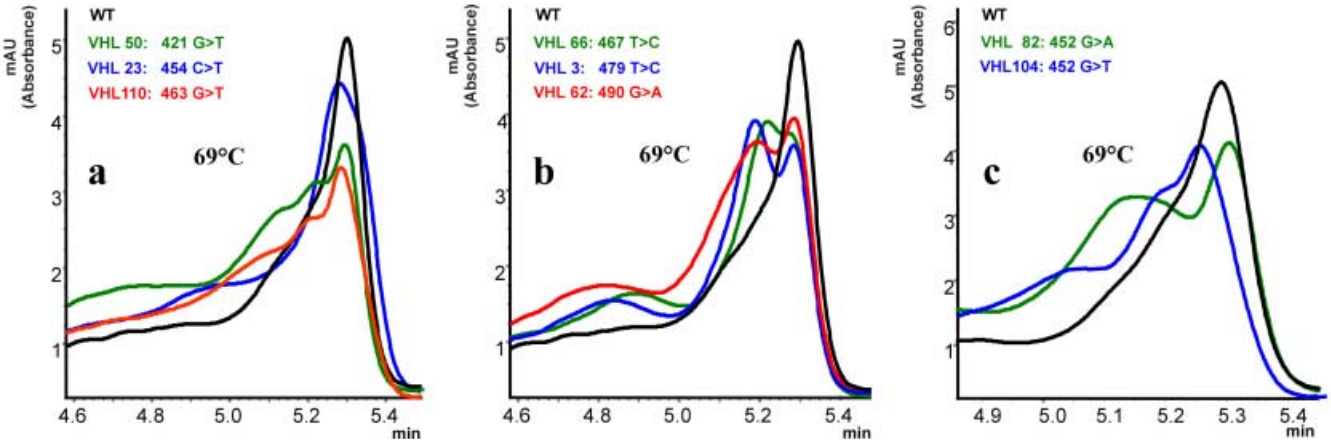

Fig. 2a–c Elution profiles of samples with *VHL* mutations of exon 1 obtained with the Wave Nucleic Acid Fragment Analysis system at 69°C. **a, b** Examples that escaped detection by the Varian-Helix system, i.e., VHL 50: 421 G→T and VHL 23: 454 C→T (**a**), and VHL 66: 467 T→C, VHL 3: 479 T→C and VHL 62: 490 G→A (**b**). Whereas the mutation VHL 23: 454 C→T remains indiscernible from the wild-type elution profile, mutation VHL 50: 421 G→T is distinguishable by the formation of peak shoulders (**a**). Mutations VHL 66: 467 T→C, VHL 3: 479 T→C, and VHL 62: 490 G→A present with a double peak mutant elution profile (**b**). Mutations VHL 82: 452 G→A and VHL 104: 452 G→T, which present with similar elution profile in the Varian-Helix system show different elution profiles with the Wave Nucleic Acid Fragment Analysis system (**c**). VHL 110: 463 G→C is a pheochromocytoma-associated VHL germline mutation that was not detected by SSCP previously (**a**)

DHPLC mutation screening of samples with unknown *VHL* germline mutations

In the series of 36 patient samples with unknown *VHL* germline mutation status, *VHL* mutations were identified in three patient samples. This identification was possible with both the Varian-Helix system and sequencing. All three mutations had previously escaped detection by Glavač et al. (1996). One frameshift mutation in a VHL patient of family VHL 73 was detected by DHPLC and identified by sequencing as an insertion C at nucleotide 396 (Fig. 1a). The other mutation (463 G→T; Val84Leu) was identified in a patient with familial pheochromocytoma of family VHL 110. This mutation was also identified in two descendants (not shown). A third mutation (490 G→C; Gly93Arg) was identified in a VHL patient of family VHL 106 (Fig. 1b).

Discussion

Eight years of international experience of *VHL* germline mutation testing has disclosed a considerable number of point mutations, small insertions, and deletions with more than 160 different mutations having been reported to the *VHL* data bases (http://web.ncifcrf.gov/research/kidney/vhlcor.html; Beroud et al. 1998; Zbar et al. 1996). A random susceptibilty to mutations becomes even more evident when somatic *VHL* mutations in clear cell renal cell carcinomas (CCRCC) are taken into account (Brauch et al. 2000; Foster et al. 1994; Gnarra et al. 1994). The combined number of reported germline and somatic *VHL* mutations is approaching 750, for a gene comprising only 639 nucleotides. Although some sites are more frequently affected than others (Brauch et al. 1995, 1999, 2000; Chen et al. 1996; Zbar et al. 1996), the cumulated published mutation data suggest that an unknown DNA sample of patients at risk may carry any *VHL* germline mutation at any nucleotide of the coding or splice consensus sequence. According to our previous experience (Glavač et al. 1996; Zbar et al. 1996; this report), some mutations were more easily identified than others. The detection of these mutations is the result of the constant improvements in mutation detection protocols, including SSCP, restriction enzyme digestion, and primer-specified restriction map modification, and in sequencing (Brauch et al. 1995; Chen et al. 1996; Glavač et al. 1996). Difficulties in the the detection of *VHL* mutations have included a lack of sensitivity, reproducibility, and reliability in SSCP analysis, an earlier frequently used mutation screening method (Orita et al. 1989). In particular, a widely distributed founder mutation in Germany affecting nucleotide 505 via a T-to-C change (Brauch et al. 1995) and a frequent hotspot mutation of European and US VHL families affecting nucleotide 712 or 713 by a C-to-T or G-to-A change, respectively (Chen et al. 1996; Zbar et al. 1996), required additional time-consuming enzymatic restriction digestion for reliable detection.

Recently, a number of reports have evaluated DHPLC as a novel DNA mutation screening method that is useful in the identification of germline mutations of cancer genes, i.e., *BRCA 1* and *BRCA 2* (Gross et al. 1999, 2000; Wagner et al. 1998, 1999a, 1999b), *TSC 2* (Choy et al. 1999) and the *MET* proto-oncogene (Nickerson et al. 2000). So far, a small number of *VHL* germline mutations have been identified by DHPLC (Ellis et al. 2000). These findings have suggested that DHPLC may be applicable in the rapid and sensitive screening of *VHL* germline mutations. In light of the multiplicity of *VHL* mutations and their distribution, a large DHPLC survey of the detection of known *VHL* mutations was needed to test the sensitivity of the method. In this study, we have subjected a panel of 43 DNA samples taken from the blood of patients with known *VHL* germline mutations to DHPLC analysis by two commercial devices and recommended protocols. Elution profiles associated with *VHL* germline mutations were established by the Varian-Helix system with an overall mutation detection rate of 86%, thus being comparable to an average SSCP result (Cotton 1993; Orita et al. 1989; Ravnik-Glavac et al. 1994; Sarkar et al. 1992). This shortcoming was mainly attributable to the *VHL* mutation detection failure rate of 33% in exon 1. It is especially important to note that the structural properties of this exon include a GC content of 70% (Herman et al. 1994), which may explain discrepancies to other studies that have reported 100% sensitivity in DHPLC mutation screening, i.e., for *BRCA 1*, *BRCA 2*, and *MET* (Arnold et al. 1999; Gross et al. 1999; Nickerson et al. 2000). Since *VHL* exon 1 comprises more than 50% of the entire *VHL* coding sequence, it is important to overcome these limitations. Following the example of others, we repeated our analyses with a Wave Nucleic Acid Fragment Analysis system and improved the detection rate to 95%. However, two mutations were not detected by either DHPLC device. One mutation in family VHL 23 is located within exon 1 at nucleotide 454 C→T, which has recently been reported to play a role in sporadic CCRCC of patients exposed to an industrial solvent (Brauch et al. 1999). There may be a future demand for the frequent and rapid screening for this particular mutation, which may not be adequately addressed by DHPLC technology. In this particular case and for the screening of individual mutations in general, it may be helpful to test different PCR primers in order to improve DHPLC sensitivity towards achieving a better ratio of the nucleotide distance between the mutation and primer location (Gross et al. 2000). The second mutation identified in family VHL 78 is located within exon 2 at nucleotide 620 T→>G, which is part of a T-rich DNA sequence. We conclude that the success rate in DHPLC-assisted *VHL* mutation detection may be influenced by different reverse-phased column technologies, by gene structure elements that impair the formation of mutation-specific conformers at calculated and tested melting temperatures, or by both.

We have also used DHPLC *VHL* germline mutation analysis for patient samples of unknown carrier status. These samples came from patients treated for ocular or

Lindau tumors and were considered to be at some risk for VHL disease by their physicians. Other samples came from individuals counseled at human genetics departments for VHL disease; however, at the time of counseling, they were free of symptoms, and family-specific mutations had not, at that time, been established in affected relatives. Samples also included patients with familial pheochromocytoma or familial CCRCC, each of which is considered to be potentially associated with VHL disease. With the exception of five cases, patients did not meet the minimal criteria for a clinical diagnosis of VHL disease (Glenn et al. 1990; Neumann 1987). Thus, the likelihood of mutation detection in most samples was low. Both DHPLC systems employed in this study detected changes in the pattern of elution profiles in the DNA of three patients who were confirmed as having *VHL* mutations by sequencing. All other DHPLC wild-type elution patterns were matched by wild-type sequencing results suggesting accurate mutation detetion by DHPLC in this panel of unknown *VHL* mutation status. Interestingly, two of the mutated samples had previously escaped detection by SSCP screening (Glavač et al. 1996). One of these mutations affected a VHL family, and the other affected a family with a history of pheochromocytoma. A third *VHL* mutation was identified in a family with clinically confirmed diagnosis of VHL disease but who had only recently come to our attention.

With respect to specificity, others have claimed that elution patterns generated by DHPLC can be used to predict the nature of mutations (Arnold et al. 1999; Gross et al. 1999; Nickerson et al. 2000). However, the DHPLC-based analysis of the *VHL* gene seems more ambivalent. On the one hand, we have observed different elution profiles for subtle changes such as different nucleotide substitutions at the same site. On the other hand, we have also observed similar elution profiles for different mutations at specific temperature increments. Thus, a prediction of specific DHPLC mutations based on the elution profile seems unlikely. This discrepancy may be explained by a greater than threefold increase in numerical ratio of mutations to total amplified sequence in this study.

In general, the Wave Nucleic Acid Fragment Analysis system seems suitable for *VHL* germline mutation screening and may also be suitable for the detection of somatic mutations in sporadic tumors. However, DNA sequencing should still be the gold standard when 100% accuracy and sensitivity are needed, as for routine *VHL* germline screening for unknown mutations. DHPLC-assisted *VHL* mutation screening may be the method of choice for testing for *VHL* carrier status in affected families for which family-specific *VHL* mutations have been identified, and for which unequivocal mutant DHPLC elution profiles have been established. This may be of interest to specialized diagnostic facilities.

Finally, it should be mentioned that 20%–30% of *VHL* germline mutations are caused by large deletions, the detection of which requires the application of quantitative Southern blotting and fluorescent in situ hybridisation analysis (Pack et al. 1999; Stolle et al. 1998).

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