

Constitutional *von Hippel-Lindau (VHL)* Gene Deletions Detected in VHL Families by Fluorescence *in Situ* Hybridization¹

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ABSTRACT

von Hippel-Lindau (VHL) disease is an autosomal dominantly inherited cancer syndrome predisposing to a variety of tumor types that include retinal hemangioblastomas, hemangioblastomas of the central nervous system, renal cell carcinomas, pancreatic cysts and tumors, pheochromocytomas, endolymphatic sac tumors, and epididymal cystadenomas [W. M. Linehan *et al.*, *J. Am. Med. Assoc.*, 273: 564–570, 1995; E. A. Maher and W. G. Kaelin, Jr., *Medicine (Baltimore)*, 76: 381–391, 1997; W. M. Linehan and R. D. Klausner, *In: B. Vogelstein and K. Kinzler (eds.), The Genetic Basis of Human Cancer*, pp. 455–473, McGraw-Hill, 1998]. The *VHL* gene was localized to chromosome 3p25–26 and cloned [F. Latif *et al.*, *Science (Washington DC)*, 260: 1317–1320, 1993]. Germline mutations in the *VHL* gene have been detected in the majority of VHL kindreds. The reported frequency of detection of *VHL* germline mutations has varied from 39 to 80% (J. M. Whaley *et al.*, *Am. J. Hum. Genet.*, 55: 1092–1102, 1994; Clinical Research Group for Japan, *Hum. Mol. Genet.*, 4: 2233–2237, 1995; F. Chen *et al.*, *Hum. Mutat.*, 5: 66–75, 1995; E. R. Maher *et al.*, *J. Med. Genet.*, 33: 328–332, 1996; B. Zbar, *Cancer Surv.*, 25: 219–232, 1995). Recently a quantitative Southern blotting procedure was found to improve this frequency (C. Stolle *et al.*, *Hum. Mutat.*, 12: 417–423, 1998). In the present study, we report the use of fluorescence *in situ* hybridization (FISH) as a method to detect and characterize *VHL* germline deletions. We reexamined a group of VHL patients shown previously by single-strand conformation and sequencing analysis not to harbor point mutations in the *VHL* locus. We found constitutional deletions in 29 of 30 VHL patients in this group using cosmid and P1 probes that cover the *VHL* locus. We then tested six phenotypically normal offspring from four of these VHL families: two were found to carry the deletion and the other four were deletion-free. In addition, germline mosaicism of the *VHL* gene was identified in one family. In sum, FISH was found to be a simple and reliable method to detect *VHL* germline deletions and practically useful in cases where other methods of screening have failed to detect a *VHL* gene abnormality.

INTRODUCTION

VHL³ disease is a familial cancer syndrome with an autosomal dominant mode of inheritance. VHL is characterized by multiple tumor types that include retinal angiomas, hemangioblastomas of the central nervous system, renal cell carcinomas, pancreatic cysts and tumors, pheochromocytomas, endolymphatic sac tumors and epididymal cystadenomas (1–3). The *VHL* gene, localized on chromosome 3p25–26, was identified as a tumor suppressor gene in 1993 (4).

Received 6/15/99; accepted 9/3/99.

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¹ This work has been funded in part by the National Cancer Institute, NIH, under Contract N01-CO-56000.

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³ The abbreviations used are: VHL, von Hippel-Lindau; FISH, fluorescence *in situ* hybridization.

The criteria for establishing VHL are based on clinical diagnosis and identification of *VHL* gene mutations. Although substantial progress has been made in defining the precise mutations causing the disease, approximately 20% of patients with clinically established VHL do not show mutations with exhaustive *VHL* gene sequencing analysis (5–9). Previous studies have shown that some of these VHL patients carried constitutional deletions of the *VHL* gene (10, 11). However, there is no simple and reliable technique to detect gene deletions in the germline screening of *VHL*.

In the past, pulse field gel electrophoresis has been used to detect VHL germline deletions (10–12). Presently, quantitative normalized Southern blot analysis is used for deletion screening (13). However, these techniques are laborious and rely on quantitative comparison of band intensities. Therefore, results can be difficult to interpret and may require confirmation by other methods. In addition, extended deletions can be missed by this technique because of the small size of the genomic probes typically used. FISH has been successfully used in gene mapping studies as well as in the identification of gene alterations, including deletions and translocations, in a variety of human diseases (14–17). This method has not been applied to the detection of constitutional *VHL* gene alterations. Advantages of FISH analysis include (a) the ability to assess individual cells; (b) detection of variable deletion sizes; (c) technical simplicity; and (d) the ability to identify deletion mosaicism. In the present study, we used FISH and genomic probes that cover the *VHL* locus and its flanking regions to analyze constitutional deletions in patients clinically diagnosed with VHL disease who did not possess *VHL* gene point mutations.

MATERIALS AND METHODS

Patients. Forty-seven individuals were included in this study (Table 1). Thirty individuals from 17 unrelated families satisfied the clinical criteria for VHL disease (18), and 6 subjects were asymptomatic offspring from 4 of these families; of the 11 individuals who were used as controls, 1 possessed a *VHL* gene point mutation, 5 had sporadic or inherited conditions other than VHL syndrome, and 5 were normal healthy donors (Table 1).

Slide Preparation. Metaphase chromosome spreads were prepared from the peripheral blood leukocytes or lymphoblastoid cell lines of each subject. Whole blood cells were grown for 72–96 h in RPMI (Life Technologies, Inc.) supplemented with 15% fetal bovine serum, penicillin-streptomycin (100 units/ml), and phytohemagglutinin (PHA) (5 µg/ml). Cells were treated with ethidium bromide (5 µg/ml) for 1.5 h, harvested after arresting with colcemid (0.05 µg/ml) for 15 min during the log-phase growth, treated in hypotonic KCl (0.54%) for 15 min at 37°C, and fixed in a cold (–20°C) methanol-acetic acid (3:1). Fresh slides were equilibrated in 2× SSC solution at 37°C and dehydrated in increasing ethanol solutions of 70, 80, and 95%.

FISH. FISH assay was performed using several probes, including P1–191 (90 kb in size) containing the entire *VHL* locus: (a) cosmid c3 (~30 kb), which includes the 3' portion of the *VHL* gene (a part of the reading frame and 3'-UTR); (b) cosmid c11 (~35 kb), which overlaps exon 1 and the 5'-UTR; and (c) the cDNA "group 7" (1.65 kb), which contains the whole VHL open reading frame and some 5'- and 3'-UTR sequences. Cosmid c3 did not

Table 1 Clinical cytogenetic data on the 47 patients and normal individuals

Number	Family	Clinical status	FISH result
1	I	VHL	Deletion P1
2		VHL	Deletion P1
3		VHL	Deletion c3, P1
4		VHL	Deletion P1
5	II	VHL	Deletion inc. ^a with P1
6	III	VHL	Deletion c11, inc. with P1
7		VHL	Deletion c11, inc. with P1
8		VHL	Deletion c11, inc. with P1
9	IV	VHL	Deletion (P1)
10	v ^b	VHL	Deletion c11/No deletion P1
11	VI	VHL	Deletion P1
12		At-risk VHL FM/asymptomatic	No deletion P1
13	VII	VHL	Deletion P1
14		At-risk VHL FM/asymptomatic	No deletion P1
15		At-risk VHL FM/asymptomatic	Deletion P1
16		VHL	Deletion P1
17		VHL	Deletion P1
18	VIII	VHL	Deletion c3/No deletion P1, c11
19	IX	VHL	Deletion c11, inc. with P1
20		VHL	Deletion c11, inc. with P1
21		VHL	Deletion c11, inc. with P1
22		VHL	Deletion c11, inc. with P1
23		At-risk VHL FM/asymptomatic	Deletion c11, inc. with P1
24		VHL	Deletion c11, inc. with P1
25	X	VHL	Mosaic (47d:53N) deletion c11, P1 inc.
26		VHL	Deletion c11, inc. with P1
27	XI	VHL	No deletion P1, c3, c11, g7
28	XII ^b	VHL	Deletion g7/No deletion P1, c11, c3
29	XIII	VHL	Deletion c11, inc. with P1
30		At-risk VHL FM/asymptomatic	No deletion P1, c11
31		VHL	Deletion c11, inc. with P1
32		VHL	Deletion c11, inc. with P1
33		At-risk VHL FM/asymptomatic	No deletion P1, c11
34	XIV	VHL	Deletion (P1)
35	XV	VHL	Deletion P1
36	XVI	VHL	Deletion c3/No deletion P1
37	XVII	Control/VHL/mutation	No deletion P1, c3, c11
38		Control/Non-VHL	No deletion P1, c3, c11
39		Control/Non-VHL	No deletion P1, c3, c11
40		Control/Non-VHL	No deletion P1, c3, c11
41		Control/Non-VHL	No deletion P1, c3, c11
42		Control/Non-VHL	No deletion P1, c3, c11
43		NL control 1	No deletion P1, c3, c11
44		NL control 2	No deletion P1, c3, c11
45		NL control 3	No deletion P1, c3, c11
46		NL control 4	No deletion P1, c3, c11
47		NL control 5	No deletion P1, c3, c11

^a inc., incomplete; FM, family member; NL, normal.

^b Family seen outside of NIH.

hybridize to group 7 cDNA (Fig. 1; Ref. 19). α -satellite centromeric probe specific for chromosome 3 (Oncor, Gaithersburg, MD) was used as a control. Our methods have been described elsewhere (20). In brief, DNA was labeled with digoxigenin-11-dUTP by nick translation (Boehringer Mannheim) and ethanol-precipitated in the presence of 50 \times herring sperm DNA and 50 \times Cot-1 human DNA. The DNA pellet was resuspended in the Hybrisol solution (50% deionized formamide/10% dextran sulfate/2 \times SSC) to a final concentration of 25 ng/ μ l. Slides were denatured in 70% formamide/2 \times SSC at 72 $^{\circ}$ C for 2 min, dehydrated sequentially in cold (-20° C) ethanol solutions of 70, 85, and 100% for 2 min and air-dried. Probes were denatured at 78 $^{\circ}$ C for 10 min

and then incubated for 30 min at 37 $^{\circ}$ C for preannealing. A total of 250 μ g of the DNA probe was applied to the slide. α -satellite repetitive DNA, specific for chromosome 3 (Oncor), was denatured separately and mixed with the cosmid probe just prior to hybridization. Overnight hybridization was done in a humidified chamber at 37 $^{\circ}$ C.

Posthybridization washes were at 45 $^{\circ}$ C in 50% formamide/2 \times SSC (5-min \times 3), /1 \times SSC (5 min \times 2) and /0.1 \times SSC (5 min \times 2). Detection was performed using avidin-FITC and antidigoxigenin Rhodamine (40 min at 37 $^{\circ}$ C) followed by washing in 4 \times SSC/0.1% Tween 20 solution at 45 $^{\circ}$ C and counterstaining with 4'6'-diaminophenylindole (DAPI)-antifade (0.25 μ g/ml).

FISH using probes from the *VHL* gene critical region in 3p25.3 was performed in a blind manner without knowing the clinical data for VHL family members. Hybridization signals were scored using a Zeiss Axiophot-2 epifluorescence microscope (Carl Zeiss, Inc., Thornwood, NY), and two-color images were captured on a Photometrics charge coupled device camera (Photometrics, Ltd., Tucson, AZ) using IPLab image software (Scanalytics Inc., Fairfax, VA). The positive normal control group displayed a 98–100% hybridization efficiency (98–100 of 100 scored cells showed positive hybridization on both homologues of chromosome 3 for P1, c3, and c11) and 73% for cDNA g7 (1.65 kb), whereas each patient showed only one homologue labeled in 98–100% of the cells. Fifty metaphases were scored while using P1, c3, and c11; and 100 cells were analyzed when g7 was used as a probe on the patients' samples. Absence of the hybridization signal in 100% of metaphases on one of the homologues of chromosome 3 was considered as a positive result for the deletion test. In case of mosaicism (Patient 25), the number of metaphases tested by using c11 was increased to 100.

RESULTS AND DISCUSSION

We first screened all 47 of the individuals using the P1 probe because it covers the entire *VHL* gene locus (Fig. 1). Partial or complete deletions were detected in 26 of the 30 VHL-disease patients (Table 1). Among 26 cases with deletions, 15 showed complete loss of one copy of the probe (Fig. 2B; Table 1), and 11 of them consistently showed significant reduction in one of two P1 hybridization signals (Fig. 2E). Retention of a very small portion of hybridized sequence in the genome may be due to the large size of the P1 probe. None of the 11 control individuals, including the patient with a known *VHL* point mutation, showed deletions using the P1, c3, c11, or g7 probes (Table 1).

To confirm the deletion status in those 11 cases with reduction of the P1 hybridization signal, we tested a cosmid clone (c11), which covers VHL intron 1, exon 1, and a 30-kb region upstream of the *VHL* gene (Fig. 1). A complete deletion was seen in all of the 11 cases with this cosmid probe (Table 1; Fig. 2F).

Four cases that showed no deletion with the P1 probe were also negative with cosmid probe c11. Cosmid probe c3, which covers the region starting about 5 kb downstream from the *VHL* polyadenylation signals and an additional 40 kb downstream, was used next (Fig. 1). We were able to detect deletion in two patients [Table 1, Patients 18 (Fig. 2C) and 36] of the four with this probe. Two patients (nos. 27 and 28), who were negative for deletions using the P1, c3, and c11 probes, were further analyzed with cDNA probe g7. One of them (Table 1, Patient 28) showed a deletion (Fig. 2D), whereas the other (Patient 27) did not. In all, 29 of the 30 individuals with VHL disease who were negative for mutations by gene sequencing were shown by FISH analysis to have moderate-to-large deletions that included the *VHL* locus.

Our study revealed a somatic mosaicism in one patient (Table 1, Patient 25) who was originally considered the unaffected mother of a *de novo* VHL patient (Table 1, Patient 26) who had been diagnosed based on the results of computed tomography scanning and FISH analysis. In this patient (No. 25), a deletion was detected in the peripheral blood leukocytes in approximately 47% of metaphases. She had been previously examined and had been considered to be negative

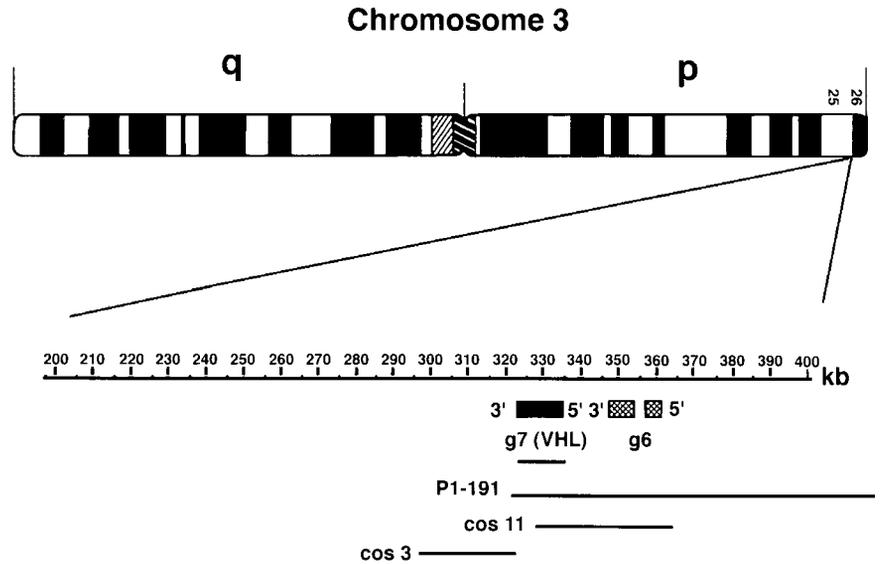


Fig. 1. Genetic map of the VHL region on 3p25.3 showing the position of the P1, c3, c11, and g7 probes in the contig encompassing the VHL locus. *p*, short arm, *q*, long arm, of chromosome 3.

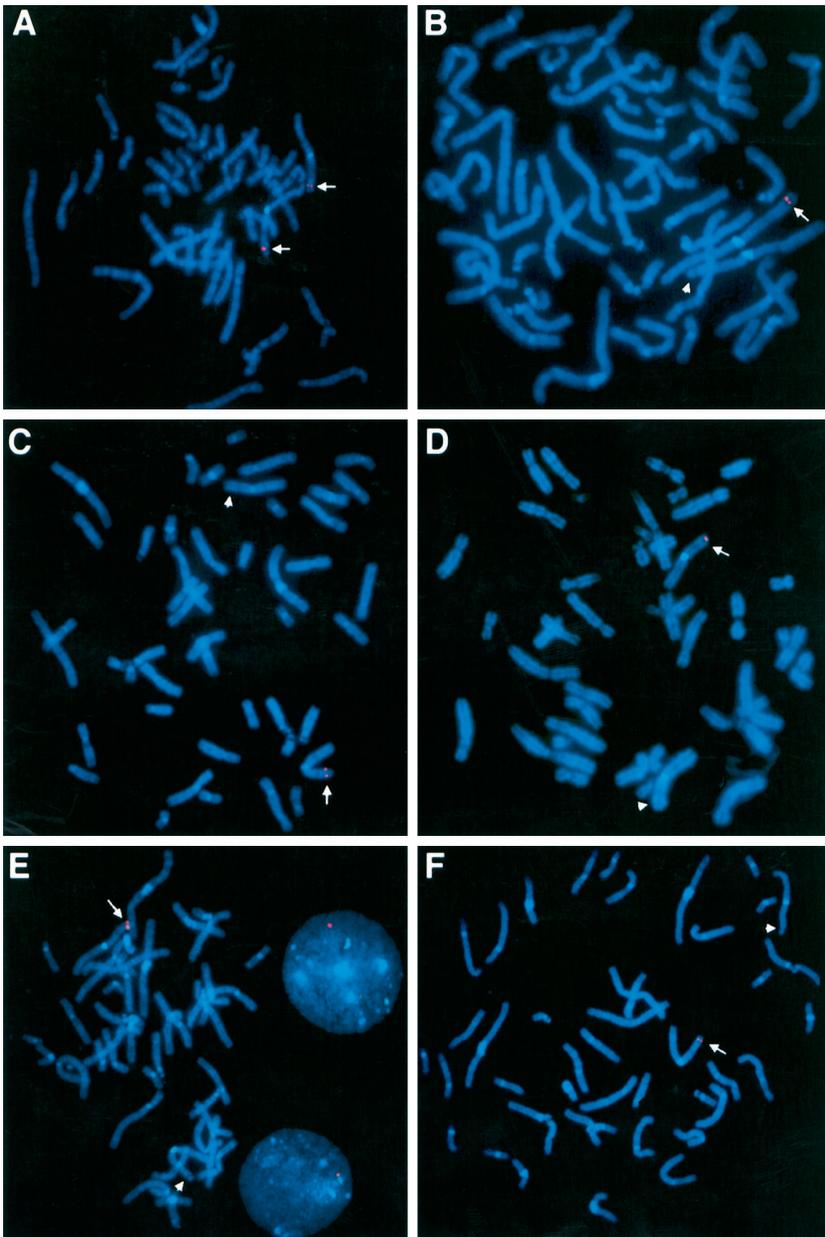


Fig. 2. Representative FISH data demonstrating allelic deletions of the VHL gene in both phytohemagglutinin-stimulated peripheral blood lymphocytes and lymphoblastoid cell lines using different probes. Rhodamine signal shows hybridization of different VHL probes; FITC signal indicates a centromeric region of chromosome 3: A, asymptomatic at-risk individual, negative for deletion (Subject 12, Family IV) using P1 probe; B, Patient 11, mother of Patient 12 (Family VI), positive for deletion using the same probe, P1; C, Patient 36 (Family XVI), showing deletion using cosmid c3; and D, Patient 28 (Family XII), positive for deletion using g7 probes. E, "incomplete" deletion detected by using the P1 probe (Patient 13, Family VII, Rhodamine signal) was further evaluated (F) with the cosmid probe c11 (Rhodamine signal) to confirm deletion status of the VHL gene. Arrows, normal homolog; arrowheads, deleted homolog of c3.

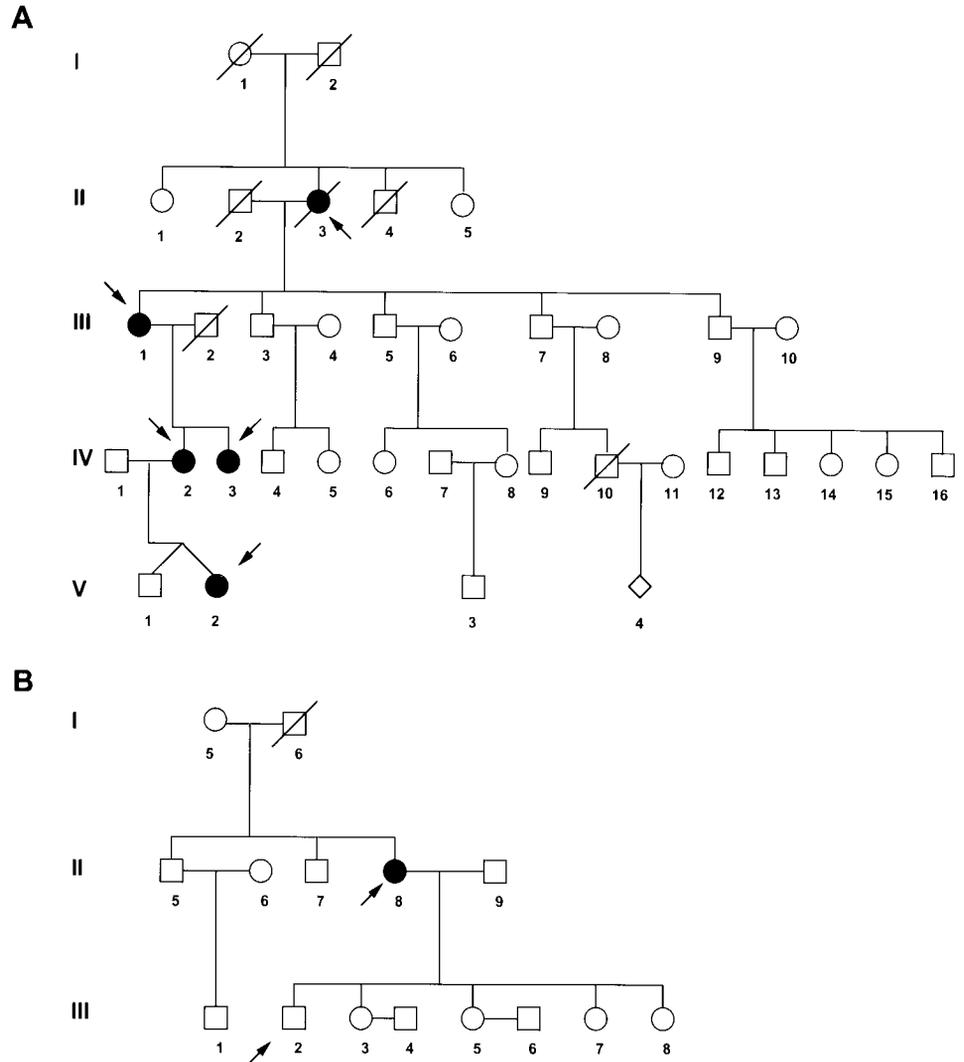


Fig. 3. Abridged pedigrees of two VHL kindreds: VII (A) and VI (B). Arrows, subjects tested by FISH. After identification of the deletion in these families, their young offspring were evaluated for the diagnosis of VHL. I-V, I-III are different generations in two families.

for germline deletion by normalized quantitative Southern blot analysis.

Constitutional deletions were identified in 29 of 30 VHL patients using genomic probes and cDNA *g7* (Table 1). In addition, six asymptomatic individuals from four independent VHL families included in this study were also screened for *VHL* gene deletions. Germline deletions were identified in two individuals and excluded in the other four. (Table 1; Fig. 3). We failed to detect a deletion in one VHL patient (Table 1, Patient 27) with all of the available probes.

Our results indicate that the extent of the deletions was variable among these VHL-disease patients; the majority of the patients possessed large deletions detectable with the P1 probe. Because there was a significant number of cases with breakpoints inside the area covered by the P1 probe, cosmid probes (~30 kb) were used to define these deletions. In one case, the deletion was small and was detected only with the cDNA probe (*g7*). Even with the use of all of the probes, no deletion was detected in one VHL patient (No. 27). This can be explained by two possibilities: (a) the deletion size was too small to be detected by any of our probes or by the FISH approach; or (b) there was a hidden mutation in the promoter area of the *VHL* gene that would be missed by routine VHL mutation screening as well.

In general, FISH offers a comprehensive way to screen for the presence of germline deletions because it enables visualization of individual cells. FISH analysis of patients with submicroscopic germline deletions and a normal karyotype may identify mosaicism or

cryptic translocation events that cannot be readily detected by other molecular strategies. An important finding of this study was the detection of an unsuspected germline deletion mosaicism in a VHL patient. FISH enabled us to identify a *de novo* mosaicism for the *VHL*-gene deletion. In this case, FISH analysis clearly demonstrated its advantage over other available methods. In addition, six asymptomatic at-risk offspring from four VHL-deletion families were evaluated by FISH screening. Two were identified as carriers of the "familial" deletion, whereas, in four others, no deletion was detected. Interestingly, two subjects (Patients 14 and 15; Fig. 3A, V:1 and V:2) were dizygotic twins from the same family: (a) one inherited the chromosome with a deletion of the *VHL* locus; and (b) the other had two apparently normal *VHL* alleles.

FISH analysis for the detection of germline deletions proved to be a useful screening method. However, the selection of the probes for screening depended on the size of deletions and the efficiency of hybridization. Whereas smaller probes may provide better coverage of the deletion spectrum, they are more likely to give a false-positive result. In our experience, the larger P1 probe provides the best reproducible hybridization efficiency, although it can miss smaller deletions because of the large size of DNA fragment covering the nondeleted area. The P1 probe, therefore, should be used in combination with smaller-size probes, such as the cosmid and cDNA probes used in this study.

FISH for the detection of constitutional allelic deletions in VHL

syndrome has the potential to improve the accuracy of current diagnostic assays and can provide important prognostic information. FISH analysis: (a) complements several other methods of gene analysis such as PCR and Southern blotting; (b) offers single-copy sensitivity; (c) permits rapid overnight analysis; and (d) uses equipment commonly found in a pathology laboratory (21).

This study demonstrates that *in situ* hybridization is an efficient method for deletion detection in VHL syndrome and may be a necessary addition to mutation screening as a routine procedure in evaluating at-risk VHL family members. It may also be useful to use with unaffected younger members of VHL as a screening and genetic counseling tool before their entering reproductive years to prevent a false sense of security obtained with negative (point) mutation blood tests.

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