

Familial Non-VHL Clear Cell (Conventional) Renal Cell Carcinoma: Clinical Features, Segregation Analysis, and Mutation Analysis of *FLCN*

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Abstract Purpose: Familial renal cell carcinoma (RCC) is genetically heterogeneous. The most common histopathologic subtype of sporadic and familial RCC is clear cell (cRCC) and von Hippel-Lindau (VHL) disease is the most common cause of inherited cRCC. Familial cRCC may also be associated with chromosome 3 translocations and has recently been described in patients with Birt-Hogg-Dube (BHD) syndrome, caused by germline *FLCN* mutation. Fewer than 20 kindreds with familial cRCC without VHL disease or a constitutional translocation have been described. The purpose of this investigation was to define the clinical and genetic features of familial non-VHL cRCC (FcRCC) and to evaluate whether unrecognized BHD syndrome might be present in patients with apparent nonsyndromic RCC susceptibility.

Experimental Design: We analyzed the clinical features of, and undertook segregation analysis in, 60 kindreds containing two or more cases of RCC (at least one confirmed case of cRCC) and no evidence of an RCC susceptibility syndrome. We also undertook *FLCN* analysis to evaluate whether unrecognized BHD syndrome might be present in 69 patients with apparent nonsyndromic RCC susceptibility.

Results: FcRCC was characterized by an earlier age at onset than sporadic cases and more frequent occurrence of bilateral or multicentric tumors. Segregation analysis showed autosomal dominant inheritance with sex- and age-dependent penetrance. A germline *FLCN* mutation was detected in 3 of 69 (4.3%) patients with apparent nonsyndromic RCC susceptibility.

Conclusions: We describe the clinical and genetic features of the largest series of FcRCC and recommend these patients be offered *FLCN* analysis, in addition to constitutional cytogenetic and *VHL* analysis.

Renal cell carcinoma (RCC) accounts for ~2% of all cancers in the Western World. RCC is histologically heterogeneous, with most (~80%) classified as clear cell (conventional; cRCC) and among the nonclear cell types, papillary (chromophilic) and

chromophobic tumors are the most frequent (1). Although only 2% to 3% of all cases of RCC are familial (2), the identification of familial cases is important to offer surveillance to patients and at risk relatives and so reduce morbidity and mortality. Familial cRCC is most commonly associated with von Hippel-Lindau (VHL) disease (MIM 193300), which is characterized by an autosomal dominantly inherited predisposition to retinal and cerebellar hemangioblastomas, cRCC, and pheochromocytoma (3, 4). The lifetime risk of cRCC in VHL disease is >70% by age 60 years (3), and annual renal imaging is offered to *VHL* mutation carriers from age 16 years. Familial cRCC may be rarely associated with constitutional translocations of chromosome 3 (5–8); however, most cases of familial cRCC not associated with VHL disease are classified as nonsyndromic (cryptogenic) FcRCC. Before 1991, just 105 patients with nonsyndromic familial RCC (9) had been reported, but molecular testing for VHL disease was not available until 1993 (4). In addition, hereditary nonclear cell papillary RCC was not well defined until 1994, and subsequently, many cases of hereditary nonclear cell papillary RCC were shown to have germline *MET* gene mutations (10–12). In the first well-defined description of FcRCC, Teh et al. (13) reported two large kindreds containing nine individuals with cRCC in whom VHL disease was excluded. Subsequently, we reported 9 families (25 affected individuals) with FcRCC

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Translational Relevance

The accurate diagnosis of inherited kidney cancer syndromes such as von Hippel-Lindau disease allows prediction of tumor risks and enables targeted surveillance to be undertaken to reduce morbidity and mortality. However, the inheritance patterns and tumor risks in patients with nonsyndromic forms of renal cell carcinoma (RCC) are not well defined. We report the clinical and genetic features of 60 kindreds containing two or more cases of RCC and no evidence of a specific RCC susceptibility syndrome. Segregation analysis was most consistent with autosomal dominant inheritance with sex- and age-dependent penetrance. These findings will facilitate genetic counseling and surveillance of families with nonsyndromic familial RCC. In addition, we investigated whether some patients with a clinical diagnosis of nonsyndromic inherited susceptibility to RCC might have unrecognized Birt-Hogg-Dube syndrome. A germline *FLCN* mutation was detected in 3 of 69 (4.3%) patients tested and we recommend these patients are offered germline *FLCN* analysis (in addition to constitutional cytogenetic and *VHL* gene mutation analysis).

(14). In contrast to the 2 families reported by Teh et al. (13) characterized by late onset RCC (8 of 9 cases age >50 years), we found an earlier mean age of onset (47.1 years) than in sporadic cases (14). Previously, Teh et al. and we considered that genetic susceptibility to cRCC was likely to be dominantly inherited (13, 14). However, in a study based on the Swedish Family-Cancer Database, it was suggested that recessive genes might be important for familial aggregation of RCC (15).

To define the clinical and genetic features of FcRCC, we analyzed a cohort of 60 kindreds containing two or more cases of RCC in close relatives (at least one of whom had histologically proven cRCC) with no evidence of VHL disease. In addition, we undertook *FLCN* mutation analysis in a cohort of patients with a genetic susceptibility to RCC to determine if another familial RCC syndrome, Birt-Hogg-Dube (BHD), might be allelic with FcRCC.

Materials and Methods

Patients. For the clinical and segregation analysis of FcRCC, 60 families with 2 or more relatives with RCC (confirmed to be clear cell in at least one individual) were ascertained via national studies of inherited RCC in Birmingham (45 families) and Paris (15 families). Patients with a clinical diagnosis of a RCC susceptibility syndrome [e.g., VHL disease, BHD syndrome (MIM 135150), and familial leiomyomatosis (MIM 605839)], germline *VHL* mutation, or constitutional translocation were excluded from the study. As DNA was not available from all probands with familial RCC included in the segregation analysis, the selection criteria for the mutation analysis study were widened so that 69 patients with features of RCC susceptibility were analyzed [28 probands from familial RCC kindreds, 22 individuals with bilateral or multicentric RCC, and 19 individuals with isolated unilateral early-onset RCC (diagnosis, <40 y)].

Segregation analysis. Segregation Analysis was done using the jPAP⁹ program. UK population incidences for RCC were obtained from the cancer diagnoses registrations for England 2004 (16).

⁹ <http://hasstedt.genetics.utah.edu/jpap/>

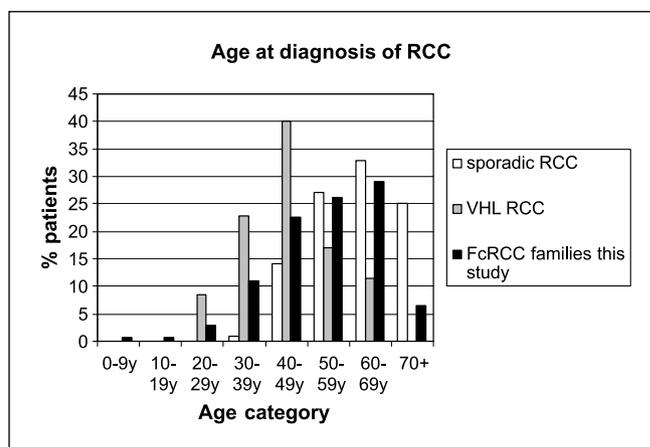


Fig. 1. Age at diagnosis of RCC in the 60 FcRCC families in this study compared with VHL disease and sporadic RCC (3, 17).

Statistical Analysis was done using Student's independent *t* test. Statistical significance was taken at 5%.

***FLCN* mutation analysis.** DNA was extracted from peripheral leukocytes using the Nucleon BACC2 kit (Amersham Biosciences). The *FLCN* gene (NM_144997.4) was screened for mutations by PCR amplification of all the coding exons and exon-intron boundaries followed by direct sequencing of PCR products. Primer sequences are available on request. PCR was done in 30- μ L volumes using 2 mmol/L magnesium chloride, 2.5 mmol/L deoxynucleotide triphosphates, and 1 unit of Taq polymerase (Invitrogen). A 10- μ L aliquot of each reaction was cleaned by addition of 10 units of Exonuclease I and 5 units of Antarctic phosphatase (New England Biolabs). The sequencing reaction was done in 10- μ L volumes using 4 μ L of cleaned PCR product, 1 \times ABI sequencing buffer, and 1 μ L Big Dye terminator cycle sequencing mix (ABI Applied Biosystems). Products were run on an ABI 3730 automated sequencer (ABI Applied Biosystems). Novel missense substitutions were verified in 280 normal control samples by restriction digestion or direct sequencing.

Results

Clinical features

The 60 families with FcRCC contained 145 affected family members (94 males and 51 females), the number of affected persons per family ranging from 2 to 5 (42 families contained two affected individuals, 13 contained three affected individuals, 3 contained 4 affected individuals, and 2 families each contained 5 affected individuals). Affected individuals were present in 3 generations in 4 families, in 2 generations in 34 families, and in a single generation in 17 families (in 12 families siblings only were affected).

The mean age at diagnosis of RCC in the 60 families was 53.2 years (SD, 14.0 years; median, 54 years; range, 9-92 years). Mean age at diagnosis of RCC in FcRCC cases was significantly younger than in sporadic cases (61.8 years; $t = 5.197$; $P < 0.0001$) but older than in patients with VHL disease (46.2 years; $t = 2.755$; $P = 0.0065$; Fig. 1; refs. 3, 17). Thirty-eight percent of patients with FcRCC were diagnosed with RCC before age 50 years compared with 71.4% of VHL patients and 15% of sporadic RCC patients (3, 17). The mean age at diagnosis of RCC in the FcRCC group was similar in males and females (53.1 years \pm 13.5 years and 53.3 years \pm 15.2 years,

respectively) and in families with just 2 affected individuals (53.2 years \pm 14.6 years) and those with 3 or more (53.1 years \pm 13.4 years) affected individuals (Fig. 2). An excess of males to females was observed in the FcrCC cohort (51 female, 94 male), similar to that observed in sporadic RCC.

Fourteen of 145 individuals (9.7%) with FcrCC were reported to have multiple tumors at the time of diagnosis or subsequently developed a second primary RCC (mean age at diagnosis of first tumor, 62 years \pm 10.8 years; range 37-81 years). Only one patient had a tumor that was diagnosed presymptomatically as a result of screening at risk relatives after a diagnosis of FcrCC within the family. All 60 kindreds contained at least 1 individual with cRCC, but in 38 families, nonclear cell RCC also occurred.

Seventeen of 145 individuals with a confirmed RCC (11.7%) also developed a nonrenal cancer or benign neoplastic lesion (Table 1). Breast cancer developed in 4 of 51 women affected by RCC and in 1 of 94 men affected with RCC. However, inspection of the 60 kindreds did not suggest that inherited susceptibility to nonrenal cancers was a feature of FcrCC.

Segregation analysis

Segregation analysis was done, and we corrected for ascertainment by conditioning in the probands and used a two allele model with age-dependent penetrance in nine age classes. Tests were at fully dominant, semidominant, recessive, and polygenic inheritance, and the -2 LN likelihood scores were 520.83, 531.56, 550.5, and 792.01, suggesting that a single gene fully dominant model with an allele frequency of 0.002 is the most likely model. Penetrance was age related (Table 2), and there was no evidence of heterogeneity between families ascertained.

FLCN mutation analysis

Mutation analysis of the *FLCN* gene of the 69 RCC patients with features of a genetic susceptibility to RCC showed 3 *FLCN* alterations (Fig. 3).

Patient 1. A novel heterozygous missense mutation (c.1213C>T; p.Arg239Cys) was detected in exon 7 of *FLCN* in a female patient who presented age 46 years with a left-sided cRCC and, at age 55 years, was found to have 5 foci of cRCC in her right kidney. Karyotype was that of normal female and

there was no family history of RCC, although it was subsequently found out that her mother had died from carcinoma secondary to a carcinoma of the descending colon. The patient was not noted to have any facial fibrofolliculomas and was not known to have any lung disease; however, she died before the detection of the *FLCN* mutation, and thus, a formal work up for the clinical features of BHD syndrome was not possible.

Although there are, as yet, no predicted functional regions of the *FLCN* protein, the encoded amino acid residue is conserved in the homologues of *FLCN* in chimpanzee, horse, dog, duck-billed platypus, mouse, rat, frog, zebra fish, mosquito, drosophila, *Caenorhabditis elegans*, and aspergillum species. Furthermore, the substitution was not detected in 280 control chromosomes and analysis by the mutation prediction programs PolyPhen¹⁰ and SIFT¹¹ showed a strong positive prediction of mutation likelihood.

Patient 2. A heterozygous frameshift mutation in exon 9 of *FLCN* (c.1388_1391delAAAG; p.Glu297AlafsX321) predicted to cause a premature stop codon was detected in a female patient who presented with an apparently isolated RCC age 25 years. There was no family history of RCC. At the time of the RCC there were no features suggestive of a diagnosis of BHD syndrome noted. However, 4 years later, review of a chest computed tomography scan undertaken at the time of renal surgery, revealed the presence of multiple pulmonary bullae.

Patient 3. A heterozygous frameshift mutation in exon 12 of *FLCN* (c.1833_1849dup17bp; p.Leu449GlnfsX473) predicted to cause a premature downstream stop codon was detected in a female patient who presented with a clear cell RCC age 28 years and had no other features suggestive of a diagnosis of BHD syndrome. Review of the family history revealed that her maternal uncle had developed a RCC age 42 years.

Discussion

Population-based case-control studies of RCC have generally suggested an increased risk of RCC in the relatives of probands with RCC (18–21). Thus, in the largest study of 1,732 RCCs an odds ratio of 1.6 for 1 first-degree relative affected was described, with 7 individuals and none of the controls reporting 2 affected relatives (20). Three other studies also reported an increased risk of RCC in relatives with odds ratios ranging from 2.5 to 5.2 (19, 21, 22). However, a potential limitation of these case-control studies is that they might include known syndromic cases of RCC susceptibility, and histopathologic subtypes of RCC are not differentiated.

Correlations between RCC histopathology and genetic susceptibility may be helpful in clinical management of familial cases. Thus, RCC associated with VHL disease is always clear cell type, hereditary nonclear cell papillary RCC associated with *MET* mutations has a type 1 papillary RCC appearance, and RCC associated with fumarate hydratase mutations (hereditary leiomyomatosis–RCC syndrome) have a distinct “type 2 papillary” RCC appearance. However, BHD syndrome is associated with a variety of RCC histopathologic subtypes

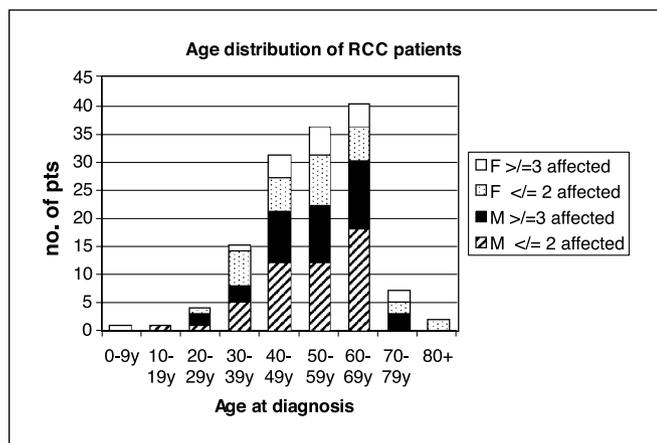


Fig. 2. Age at diagnosis of RCC in males and females from families with less than or equal to two affected family members and families with greater than or equal to three affected family members.

¹⁰ <http://genetics.bwh.harvard.edu/pph/>

¹¹ <http://blocks.fhrc.org/sift/SIFT.html>

Table 1. Details of 19 lesions, either nonrenal cancers or benign neoplastic lesions occurring in 17 of 145 FcRCC patients

Family ID	Sex	Age at diagnosis of RCC (y)	RCC details	Other tumor and age at diagnosis
A	M	63	cRCC (two primary tumors)	Breast cancer, 65 y
B	F	54	cRCC (bilateral)	Breast cancer, 51 y
C	F	58	RCC	Breast cancer, 55 y
D	F	60	cRCC	Benign adrenal cortical adenoma, 58 y
E	F	47	cRCC	Basal cell carcinoma, 32 y; uterine leiomyomas, 30 y
F	F	37	cRCC (bilateral)	Breast cancer, 41 y
G	M	54	cRCC (bilateral)	Prostate cancer, 61 y
H	F	50	oncocytoma	Bladder cancer, 52 y
J	F	70	cRCC	Basal cell carcinoma, 67 y; colon polyps, 72 y
J	M	41	cRCC	Basal cell carcinoma, 48 y
K	F	69	cRCC	Breast cancer, 68 y
L	F	54	cRCC	Cervical cancer, 55 y
M	M	49	cRCC	Adrenal adenoma, age unknown
N	F	56	RCC	Uterine leiomyomas, 56 y
N	F	33	cRCC	Uncharacterized thyroid nodules, 26 y

Abbreviations: F, female; M, male.

including cRCC, chromophobe RCC, and a hybrid oncocytic neoplasm with areas of both chromophobe RCC and oncocytoma (23).

We have described the largest cohort of nonsyndromic FcRCC and have done the first segregation analysis of this condition. Segregation analysis was most consistent with a model of autosomal dominant inheritance. In larger families (3 or more affected individuals) inspection of the pedigree is usually consistent with dominant inheritance, but in small families (with only 2 affected individuals) only siblings were affected in 26.2%. Previously, two epidemiologic studies reported higher risks of RCC in siblings than in parents of affected individuals (although this was only statistically significant in one study), suggesting a recessive mode of inheritance (15, 22). Within our cohort, we identified 12 families with only affected siblings and none of these were consanguineous. FcRCC shows evidence of incomplete and age-dependent penetrance and so, although recessive inheritance cannot be excluded in a subset of patients with FcRCC, most cases of FcRCC are likely to be dominantly inherited. The genetic significance of small families with only two affected relatives with RCC may be difficult to evaluate as such families might occur by chance or due to shared environmental factors. However, we note that the mean age at diagnosis of RCC in these small families (53.0 years) was younger than in sporadic cases and similar to that in families with 3 or more affected individuals (53.1 years), suggesting that such cases frequently have a genetic basis.

The introduction of annual renal imaging for patients and relatives at risk of VHL disease has led to the detection of RCC at an earlier age when they are usually asymptomatic (24). However, in our cohort of 60 FcRCC families, only 1 of 145 affected patients had a RCC diagnosed presymptomatically. Based on our experience of FcRCC, we recommend that annual renal imaging (ultrasound or magnetic resonance imaging) is offered to affected patients and relatives from age 30 years. Fewer than 5% of RCC in our FcRCC cohort were diagnosed before 30 years, but in families with earlier onset tumors, earlier surveillance should be offered. Although many

familial cancer syndromes are characterized by susceptibility to multiple tumor types, we did not find convincing evidence of susceptibility to non-RCC site-specific cancers in most FcRCC kindreds.

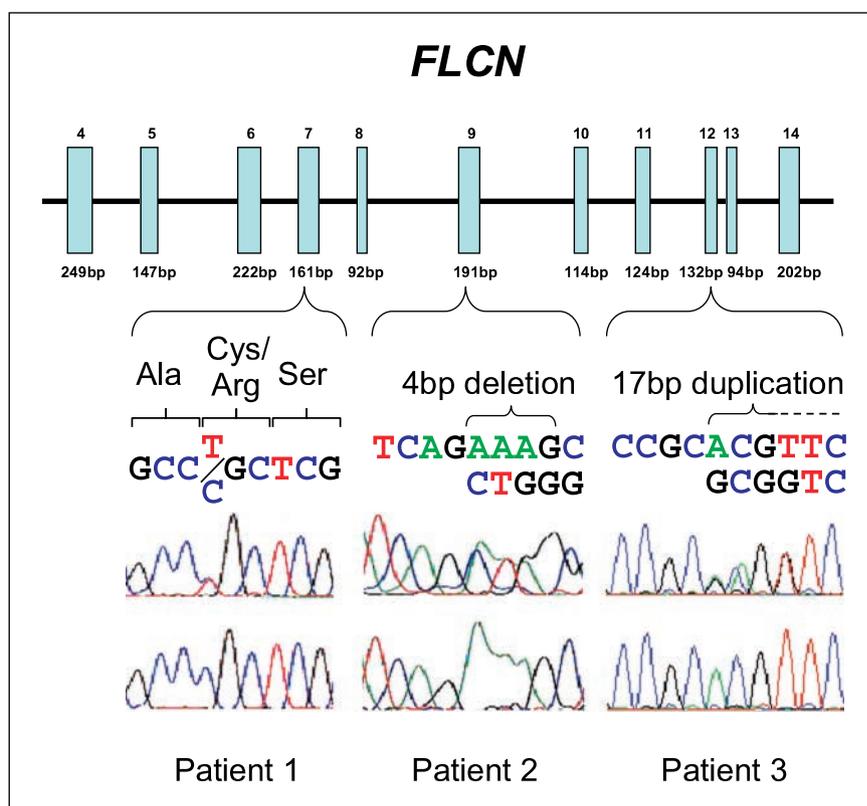
The identification of the molecular basis for FcRCC would facilitate diagnosis and allow screening to be targeted to high-risk individuals. In our experience, patients presenting with FcRCC will be found rarely to have a germline *VHL* mutation or constitutional translocation, and the majority of patients will have cryptogenic FcRCC (14, 25). However, the frequency of *VHL* mutation detection will depend on the extent to which individuals are screened for clinical and subclinical (e.g., asymptomatic lesions detected by magnetic resonance imaging scanning) lesions. In one study, *VHL* disease was present in 1.6% of unselected RCC patients, and clearly, it is important that patients with features of inherited RCC susceptibility are investigated for underlying *VHL* disease (26). Interestingly, although specific *VHL* missense mutations may cause a pheochromocytoma only phenotype (type 2C *VHL* disease), no *VHL* mutations have yet been associated with a RCC-only phenotype (27).

Table 2. Segregation analysis

Age (y)	Penetrance (%)	
	Males	Females
5	1.3	1.4
15	4.1	4.4
25	7.2	7.8
35	12.0	12.9
45	23.8	22.1
55	46.3	38.4
65	91.7	55.9
75	93.0	69.2

NOTE: Age-dependent penetrance estimates for dominant model (estimated allele frequency, 0.002).

Fig. 3. Schematic diagram of coding region of FLCN (NM.144997.4) and electropherograms showing FLCN mutations detected (c.1213C>T; p.Arg239Cys, c.1388.1391delAAAG; p.Glu297AlafsX321 and c.1833.1849dup17bp; p.Leu449GlnfsX473) in three patients with a genetic susceptibility to RCC.



The identification of an underlying RCC susceptibility syndrome allows relatives to be screened and reduces morbidity. In view of this, we speculated whether another dominantly inherited RCC susceptibility syndrome, namely BHD, could present as FcRCC. BHD is characterized by cutaneous fibrofolliculomas and pneumothorax secondary to pulmonary bullae (28–30). The occurrence of colorectal polyps in gene carriers has also been noted in some families (31). Expression of BHD is variable and previously we observed a kindred with FcRCC in which additional features of BHD subsequently became apparent.¹² We therefore investigated the frequency of germline *FLCN* mutations in a cohort of 69 patients with features of RCC susceptibility. Three patients, ~4% (upper 95% confidence interval for population frequency, 9.16%) of patients had putative mutations. A frameshift truncating mutation was detected in a woman who presented with RCC age 25 years. Although no features of BHD were noted at that time, it was subsequently noted that she had had multiple pulmonary bullae. A further frameshift truncating mutation was detected in female patient who developed a clear cell RCC age 28 years. There was a family history of RCC with the condition affecting her maternal uncle age 42 years. In addition, a p.Arg239Cys missense substitution was identified in a female patient who developed multicentric cRCC age 55 years after a contralateral cRCC 9 years previously. This novel missense substitution occurred at a highly conserved residue and was not detected in control samples and, although *FLCN* missense mutations seem to be rare (32), it seems likely that

this is pathogenic mutation. As the patient had died, it was not possible to reinvestigate for subclinical evidence of extrarenal features of BHD.

We note that *FLCN* mutations have been reported not only in BHD but also in familial pneumothorax without other features of BHD (33, 34). Hence, we cannot exclude the possibility that the p.Arg239Cys mutation might predispose to RCC but not other features of BHD. We note that a missense mutation at codon 255 (p.His255Arg) in the canine *FLCN* orthologue causes hereditary multifocal renal cystadenocarcinoma and nodular dermatofibrosis in German shepherd dogs (35). Mutation analysis of *FLCN* in sporadic RCC cell lines and tumors has generally shown that *FLCN* mutations are infrequent, but a somatic missense mutation (p.Ala444Ser) with loss of the wild-type allele was detected in a primary cRCC and a p.Ala238Val missense mutation (adjacent to the codon mutated in Patient 1) was identified in a clear cell RCC cell line for which matched normal DNA was not available (36). However, no missense mutations were detected in any of the eight somatic *FLCN* mutations detected in a series of chromophobe RCC and oncocytomas (37). Although the folliculin protein has been linked to the AMPK and mammalian target of rapamycin pathways, folliculin function has not been characterized in detail and so it is not possible to predict how mutations in specific regions of folliculin might effect on folliculin function(s) and related these to possible genotype-phenotype correlations.

Nevertheless, it is clear that rare patients presenting with apparent nonsyndromic RCC susceptibility may subsequently prove to have BHD syndrome, and so we recommend that patients with evidence of possible FcRCC should, in addition to clinical evaluation for an underlying RCC susceptibility

¹² E.R. Woodward and E.R. Maher, unpublished observations.

syndrome and cytogenetic and germline *VHL* analysis, be offered *FLCN* mutation analysis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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