

## Germline *BHD*-Mutation Spectrum and Phenotype Analysis of a Large Cohort of Families with Birt-Hogg-Dubé Syndrome

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Birt-Hogg-Dubé syndrome (BHD), a genodermatosis characterized by multiple hamartomas of the hair follicle (fibrofolliculoma), predisposes individuals to an increased risk of developing renal neoplasms and spontaneous pneumothorax. Previously, we localized the *BHD* locus (also known as *FLCN*) to chromosome 17p11.2 by linkage analysis and subsequently identified germline mutations in a novel gene in probands from eight of the nine families with BHD in our screening panel. Affected members of five of the families inherited an insertion/deletion of a cytosine in a C<sub>8</sub> tract in exon 11. This mutation was also identified by exon 11 screening in probands from 22 of 52 additional families with BHD and therefore represents a hypermutable “hotspot” for mutation in *BHD*. Here, we screened the remaining 30 families from this large BHD cohort by direct sequence analysis and identified germline *BHD* mutations in 84% (51/61) of all families with BHD recruited to our study. Mutations were located along the entire length of the coding region, including 16 insertion/deletion, 3 nonsense, and 3 splice-site mutations. The majority of *BHD* mutations were predicted to truncate the BHD protein, folliculin. Among patients with a mutation in the exon 11 hotspot, significantly fewer renal tumors were observed in patients with the C-deletion than those with the C-insertion mutation. Coding-sequence mutations were not found, however, in probands from two large families with BHD whose affected members shared their family’s BHD-affected haplotype. Of the 53 families with BHD whose members inherited either a germline mutation or the affected haplotype, 24 (45%) had at least one member with renal neoplasms. Three families classified with familial renal oncocytoma were identified with *BHD* mutations, which represents the first disease gene associated with this rare form of renal neoplasm. This study expands the *BHD*-mutation spectrum and evaluates genotype-phenotype correlations among families with BHD.

### Introduction

Birt-Hogg-Dubé syndrome (BHD [MIM 135150]) was first described in 1977 by three Canadian physicians who studied a large family whose members were affected with multiple, small papules on the face, neck, and upper torso that developed after age 25 years (Birt et al. 1977). These lesions, called “fibrofolliculomas” (FFs), were characterized as benign hamartomas of the hair follicle. His-

tologically, FFs are characterized by strands of proliferating epithelial cells extending out from the infundibulum of the hair follicle, surrounded by a thick mantle of connective tissue and mucin-rich stroma (Birt et al. 1977). FFs are part of the triad of BHD skin lesions that also includes trichodiscomas and acrochordons. BHD is inherited in an autosomal dominant manner and has been reported to be associated with renal tumors (Roth et al. 1993; Toro et al. 1999), spontaneous pneumothorax (Binet et al. 1986; Toro et al. 1999), colon polyps, and colon carcinomas (Hornstein and Knickenberg 1975; Hornstein 1976; Rongioletti et al. 1989; Le Guyadec et al. 1998). A recent risk-assessment study of BHD-affected patients concluded that a diagnosis of BHD conferred a 7-fold increased risk of developing renal neoplasia and a 50-fold increased risk of spontaneous pneumothorax but no increased risk of colon polyps or colon cancer (Zbar et al. 2002). Renal tumors with a variety

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of histologies are associated with BHD, including chromophobe renal cell carcinoma (RCC), benign renal oncocytoma, clear-cell RCC, and papillary type I RCC (Pavlovich et al. 2002, 2005). The most common form of renal tumor seen in patients with BHD is an oncocyctic hybrid tumor with areas reminiscent of both chromophobe RCC and oncocytoma (Tickoo et al. 1999).

The BHD disease locus was mapped to chromosome 17p11.2 by genetic linkage analysis (Khoo et al. 2001; Schmidt et al. 2001); we subsequently identified germline mutations in a novel gene (*BHD* [GenBank accession number AF517523], or *FLCN* [MIM 607273]) in a panel of nine kindreds with BHD. These were insertions, deletions, and nonsense mutations that were predicted to truncate the BHD protein, folliculin (Nickerson et al. 2002). A majority of kindreds with BHD were found to harbor an insertion or deletion of a cytosine in a C<sub>8</sub> tract within exon 11, suggesting a hypermutable “hotspot” for mutation in *BHD* (Khoo et al. 2002; Nickerson et al. 2002).

Folliculin, is a 579-aa protein with no known functional domains, for which mouse, fly, worm, yeast, dog, and rat orthologs have been identified (Nickerson et al. 2002; Lingaas et al. 2003; Okimoto et al. 2004). *BHD* mRNA expression, measured by fluorescent in situ hybridization, is widespread in a variety of tissues, including skin and its appendages, the distal nephron of the kidney, and stromal cells and type I pneumocytes of the lung (Warren et al. 2004). Strong *BHD* mRNA expression was found in secretory cells, such as acinar cells of the parotid gland and pancreas, and ductal cells of the breast. Reduced *BHD* expression was seen in renal tumors from patients with BHD, regardless of histologic type.

Sixty-one families affected with BHD were recruited to the NCI for study over a 3-year period. Previously, we evaluated a screening panel representing nine of these families with BHD and reported the identification of one nonsense and two frameshift *BHD* mutations as well as five insertion/deletion mutations in the C<sub>8</sub> tract of exon 11. Exon 11 screening of the entire cohort of families revealed C<sub>8</sub> tract insertion/deletion mutations in probands from 22 of the 52 remaining families with BHD (Nickerson et al. 2002). In the present study, we have completed the mutation analysis of this large BHD cohort by screening, by direct sequence analysis, the remaining 30 families for mutations in the *BHD* gene. We have identified germline *BHD* mutations in affected members of 84% (51/61) of kindreds with BHD evaluated to date. In addition, we have collected phenotypic information on family members and have correlated phenotype with germline *BHD* mutation to evaluate possible genotype-phenotype associations.

## Methods

### Patient Recruitment

We recruited members of 61 BHD-affected families to our study at the NCI, from 1998 to 2001, through patient recruitment letters to dermatologists (55 families) and by referrals from urologic surgeons (6 families). All of the families with BHD were invited to participate in the study regardless of the number of affected individuals in the family or the presence or absence of associated health problems. A family was considered affected with BHD if it had (1) one or more members with 10 or more skin lesions that were clinically compatible with FFs and/or (2) a minimum of one histologically proven FF. Histologically, an FF was characterized by multiple anastomosing strands of 2–4 epithelial cells extending from a central hair follicle. Phenotypic expression of BHD skin papules can be variable among affected members of a family with BHD; therefore, once a proband with clinically positive or histologically proven FFs was identified in a BHD-affected family, other family members were screened and classified as affected for genotype-phenotype evaluation on the basis of (1) the presence of a histologically proven FF, (2) inheritance of the family's germline *BHD* mutation, (3) inheritance of the family's BHD-affected haplotype, or (4) obligate carrier status. We also included family 238 as affected with BHD, because multiple members were affected with bilateral, renal oncocyctic hybrid neoplasms, a rare histologic variant uniquely associated with BHD. Participants in this study provided written informed consent. The protocol was approved by the institutional review boards of the NCI and the University of Manitoba.

### Patient Evaluation

All members of families with BHD who were aged >20 years were evaluated at the NIH Clinical Center and/or in the field. Blood samples were obtained for DNA extraction and mutation analysis. Each patient received a detailed dermatologic examination, and biopsies were performed for lesions suspected to be FFs.

Family members seen at the NIH were evaluated for other phenotypic manifestations associated with BHD. Occult renal malignancies were detected by CT scan of the abdomen before and after administration of ~120 ml of Ioxilan 300 (Cook Imaging). The presence of lung cysts and evidence of spontaneous pneumothorax were evaluated by thoracic CT scan, by use of high-resolution sections (1 mm) through the chest at 10-mm intervals. Additional information was obtained from patient medical histories, outside medical records, and tissue specimens obtained for NIH pathology review.

### *Mutation Analysis of the BHD Gene*

DNA was extracted from peripheral-blood leukocytes, in accordance with standard procedures. Exon/intron structure of the *BHD* gene was determined by BLAST alignment of two overlapping, uncharacterized, full-length transcripts from skin melanoma (GenBank accession numbers BC015725 and BC015687), against the BAC clone RP11-45M22 (GenBank accession number AC055811); intronic primers were designed to amplify 14 coding exons and splice junctions for sequencing, as described elsewhere (Nickerson et al. 2002). Subsequent analyses showed that this exon/intron structure matched the cloned full-length *BHD* transcript. PCR products were generated using standard conditions and were purified; double-stranded sequencing was performed using BigDye Terminators (Applied Biosystems), as described elsewhere (Nickerson et al. 2002). Sequence variants were examined for cosegregation with disease in their respective families by denaturing high-performance liquid chromatography or by single-stranded sequencing. Insertions and deletions were subcloned with Topo Cloning Kit (Invitrogen) and were sequenced. At least 160 unaffected individuals were examined for each disease-associated sequence variant.

### *BHD Haplotype Carrier Analysis*

Family members were evaluated for inheritance of the BHD-affected haplotype in cases in which a *BHD* germline mutation was not identified in the proband. To determine if a family member inherited the BHD-affected haplotype, individuals were genotyped using seven microsatellite markers (*D17S953*, *D17S740*, [location of *BHD*] *D17S2196*, *D17S620*, *AGAT100*, *D17S805*, and *D17S1824*) that spanned an 8-cM distance flanking the *BHD* locus on chromosome 17p. Haplotypes were determined from the genotype order in which the least number of recombinants occurred. All members of BHD-affected families who presented with FFs inherited the family's BHD-affected haplotype.

### *Statistical Analysis*

The percentage of BHD-affected individuals with renal tumors was calculated in two ways: (1) by dividing the number of BHD-affected individuals with renal tumors by the number of BHD-affected individuals seen at the NIH and in the field, and (2) by dividing the number of BHD-affected individuals who had renal tumors by the number of BHD-affected individuals who were screened by abdominal CT scan. To overcome selection bias, we assessed the frequency of renal tumors found in patients with BHD two ways. Generally, patients who were affected with BHD skin lesions and/or were symptomatic for renal tumor came to the NIH to

be seen and screened. Some patients who were less severely affected did not come to the NIH and were seen only in the field and therefore did not undergo abdominal/pulmonary CT screening. Differences in frequency of renal tumors, pneumothorax, or lung cysts between the groups with exon 11 C-insertion and C-deletion mutations were tested with Fisher's exact test (two-sided) (see the Calculation of Fisher's Exact Test Web site).  $P < .05$  was considered statistically significant.

## **Results**

### *Description of Patients*

For the present study, 219 individuals (110 females and 109 males) from 53 families (whose members inherited either a germline mutation or the affected haplotype) were classified as affected with BHD. Of those individuals, 187 were evaluated in the field or at the NIH Clinical Center. Data were available for 7 deceased individuals with obligate carrier status, 12 individuals were evaluated by outside dermatologists, and 10 individuals provided a blood sample for mutation analysis but did not undergo further medical evaluation at the NIH.

The number of affected individuals in a family ranged from 1 to 25. In the present study, the largest number (25) of affected family members in a family with BHD was found in the Canadian family with BHD, family 172, originally studied by Drs. Birt, Hogg, and Dubé (Birt et al. 1977). Twenty-four families had only one member affected with BHD.

### *Dermatologic Manifestations*

The hallmark BHD cutaneous manifestation is a benign tumor (hamartoma) of the hair follicle, an FF. Of the 175 BHD-affected individuals whose skin papules were biopsied and evaluated histologically, 147 (84%) were diagnosed with a histologically proven FF. All families that carried a *BHD* mutation or inherited the affected haplotype contained individuals who developed BHD skin lesions, with the exception of one family, family 238, referred to the NCI by the proband's urologist, in which three sisters developed bilateral renal oncocytic hybrid neoplasms and lung cysts without evidence of FFs. In the present study, the cutaneous lesions were not found in patients aged <20 years.

### *Pulmonary Manifestations*

A high percentage of patients with BHD develop air-filled lung cysts (Zbar et al. 2002) and have a 50-fold higher risk of developing spontaneous pneumothorax. In the present study, 64 (32%) of 198 BHD-affected individuals for whom medical history was available had a history of spontaneous pneumothorax. Of the 129

BHD-affected individuals who were screened by pulmonary CT scan, 110 (85%) were found to have one or more lung cysts consistent with the BHD phenotype. Forty-five (85%) of 53 families with *BHD* germline mutations or affected-haplotype carriers had members who developed lung cysts or spontaneous pneumothorax.

#### *Renal Neoplasia in BHD*

BHD is unique among inherited renal cancer syndromes because patients develop not one, but a variety of histologic types of renal tumors, including chromophobe RCC, renal oncocytoma, clear-cell RCC, and papillary RCC (Pavlovich et al. 2002, 2005). Most often, BHD-associated renal neoplasms are oncocytic hybrid tumors, comprising elements of both chromophobe and renal oncocytoma. Thirty-eight BHD-affected individuals representing 24 BHD-affected families developed renal tumors. Males developed renal tumors 2.5-fold more often than did females (27 males; 11 females). The median age at diagnosis was 48 years (range 31–71 years). Tumors developed both bilaterally with multiple foci and unilaterally with a single focus. The number of patients with renal neoplasia in a BHD-affected family ranged from one to three, most frequently one case per family. The frequency of renal tumor among BHD-affected individuals seen at the NIH or in the field was 20% (38/187). The frequency of renal tumor among BHD-affected individuals who were evaluated by CT scan was 29% (38/132). Overall, 45% (24/53) of BHD-affected families with germline *BHD* mutations or affected-haplotype carriers contained individuals who were affected with renal tumors.

#### *Other Phenotypic Features Found in BHD-Affected Individuals*

There are reports that describe a number of additional phenotypic manifestations in BHD-affected individuals, including colon polyps and colon carcinoma (Hornstein and Knickenberg 1975; Hornstein 1976; Rongioletti et al. 1989; Le Guyadec et al. 1998), lipomas (Chung et al. 1996; Toro et al. 1999), angioliomas (Chung et al. 1996), parathyroid adenomas (Chung et al. 1996), and parotid oncocytomas (Liu et al. 2000). Among the 219 BHD-affected individuals in the present study, four parotid gland tumors were reported in 2 men (diagnosed at ages 20 and 39 years) and 2 women (diagnosed at ages 62 and 72 years). Three of the parotid tumors were classified as oncocytomas. Additionally, three individuals with lipomas and nine individuals with colon polyps/carcinoma were identified through medical history.

#### *Variable Expression of Phenotypic Manifestations*

BHD is characterized by FFs, lung cysts, spontaneous pneumothorax, and an increased risk of developing renal

neoplasia. These manifestations may occur singly or in various combinations in a patient, and variable phenotypic expression may be observed among family members from the same BHD-affected family. Among the 53 families with BHD whose affected members had a *BHD* germline mutation or carried the BHD-affected haplotype, 22 (41.5%) of the families had skin, lung, and renal manifestations, 22 (41.5%) of the families had members with skin and lung phenotypic features, 1 (2%) of the families had renal and lung phenotypic features, 7 (13%) of the families had only skin papules, and 1 (2%) of the families had renal tumors and skin papules (table 1).

#### *BHD-Mutation Spectrum and Mutational Hotspot*

Elsewhere, we reported the identification of *BHD* germline mutations, by direct sequence analysis, in 30 of 61 families with BHD recruited to the NCI (Nickerson et al. 2002). In the present study, we have completed mutation analysis of the remaining 30 families and, to date, have identified germline *BHD* mutations in 51 (84%) of 61 families with BHD in the NCI cohort. Each family's mutation cosegregated with disease and was not found in at least 160 unaffected individuals. Altogether, 22 novel mutations have been identified (5 were reported elsewhere [Nickerson et al. 2002]), including 16 insertions or deletions that cause frameshifts leading to premature truncation of the protein, 3 nonsense mutations that encode early termination codons, and 3 putative splice-site mutations (table 1 and fig. 1). Of note, no germline missense *BHD* mutations were detected in these BHD-affected families.

A total of 53% (27/51) of families with a germline *BHD* mutation had a cytosine insertion (c.1733insC [18 families]) or deletion (c.1733delC [9 families]) in the mononucleotide tract of eight cytosines (c.1733–1740) in exon 11. This mutational hotspot has been identified by several laboratories (Khoo et al. 2002; Nickerson et al. 2002). Mononucleotide tracts are thought to be hypermutable due to slippage of the DNA polymerase during replication (Streisinger et al. 1966), which results in frameshift mutations leading to protein truncation. Although most of the mutations identified in the remaining 24 BHD-affected families were unique, 2 families had the same 28-bp duplication in exon 9 (c.1378–1405 dup), 3 families shared a nonsense mutation in exon 12 (c.1844C→G), and 2 families shared a putative splice-site mutation in intron 9 (IVS9+2T→G) (table 1 and fig. 1).

#### *Genotype-Phenotype Correlation: Location within the BHD Gene*

Mutations were identified in all translated exons (exons 4–14), with the exception of exons 8 and 10 (fig. 2). Exon 11 was the most frequent site of mutation in

**Table 1**

**BHD Germline Mutations and Phenotypes of Families with BHD**

FAMILY	MUTATION ANALYSIS				NO. OF FAMILY MEMBERS WITH PHENOTYPE				
	Exon	Nucleotide <sup>a</sup> Change	Codon <sup>b</sup> Location	Type <sup>c</sup> (aa→ter)	Total Affected	FF <sup>d</sup>	Renal Tumors	Lung Cysts <sup>e</sup>	PT <sup>f</sup>
220	4	c.513delT	F20	FS (34)	1	1 <sup>g</sup>	0	ND	0
249	5	IVS4-1G→A	G84	SS	3	1	1	ND	2
8188	5	c.707delC	G84	FS (45)	2	2	0	2	0
209	5	c.751delA	D99	FS (30)	2	2	0	1	0
233	6	c.1036delG	G195	FS (27)	1	1	0	1	0
202	7	c.1087delAGinsC	E211	FS (11)	8	7	1	5	0
224	7	c.1092delT	F213	FS (9)	1	1	0	1	0
166	7	c.1126delCA	T224	FS (22)	4	4	3	4	0
217	9	c.1378-1405dup	T317	FS (79)	1	1	1	1	0
228	9	c.1378-1405dup	T317	FS (79)	12	11	2	7	4
231	9	c.1468delG	W338	FS (14)	2	2	0	2	1
225	9	c.1473delC	R341	FS (11)	1	1 <sup>g</sup>	ND	ND	0
242	9	IVS9+1G→A	V355	SS	9	6	1	7	8
229	9	IVS9+2T→G	V355	SS	5	5	3	4	1
238	9	IVS9+2T→G	V355	SS	3	0	3	3	0
239	11	c.1733insC	H429	FS (26)	1	1	1	1	1
8244	11	c.1733insC	H429	FS (26)	1	1 <sup>g</sup>	0	1	0
8319	11	c.1733insC	H429	FS (26)	1	1	0	1	0
7561	11	c.1733insC	H429	FS (26)	1	1 <sup>h</sup>	ND	ND	0
187	11	c.1733insC	H429	FS (26)	1	1	1	1	0
7399	11	c.1733insC	H429	FS (26)	1	1	0	0	0
250	11	c.1733insC	H429	FS (26)	3	2	1	1	1
246	11	c.1733insC	H429	FS (26)	8	8 <sup>g</sup>	0	0	2
191	11	c.1733insC	H429	FS (26)	1	1 <sup>h</sup>	ND	ND	0
211	11	c.1733insC	H429	FS (26)	1	1	0	1	0
241	11	c.1733insC	H429	FS (26)	4	4	1	4	0
183	11	c.1733insC	H429	FS (26)	1	1	1	ND	1
208	11	c.1733insC	H429	FS (26)	1	1	0	1	0
167	11	c.1733insC	H429	FS (26)	3	1	2	3	2
210	11	c.1733insC	H429	FS (26)	11	6	0	2	6
174	11	c.1733insC	H429	FS (26)	10	8	3	9	0
216	11	c.1733insC	H429	FS (26)	16	11	2	8	8
200	11	c.1733insC	H429	FS (26)	7	3	1	2	4
232	11	c.1733delC	H429	FS (38)	5	4	0	4	0
248	11	c.1733delC	H429	FS (38)	3	2	ND	ND	1
234	11	c.1733delC	H429	FS (38)	2	2	0	2	2
176	11	c.1733delC	H429	FS (38)	1	1	0	1	1
226	11	c.1733delC	H429	FS (38)	1	1	0	0	0
175	11	c.1733delC	H429	FS (38)	1	1	0	1	0
184	11	c.1733delC	H429	FS (38)	1	1	1	1	0
201	11	c.1733delC	H429	FS (38)	6	5	0	4	0
185	11	c.1733delC	H429	FS (38)	7	3	ND	1	5
6932	12	c.1758delT	F435	FS (32)	1	1	1	1	1
7416	12	c.1834delTTC	L460	FS (24)	1	1	0	1	0
230	12	c.1844C→G	Y463X	NS	9	7	1	2	2
188	12	c.1844C→G	Y463X	NS	2	2	0	2	1
245	12	c.1844C→G	Y463X	NS	2	2	0	ND	1
8015	12	c.1881insG	D476	FS (9)	1	1	1	ND	0
7707	12	c.1884C→T	R477X	NS	1	1 <sup>g</sup>	ND	ND	1
7706	13	c.1945insCTGT	V497	FS (24)	1	1 <sup>g</sup>	ND	ND	0
168	14	c.2034C→T	R527X	NS	5	1	3	3	1
172	NF <sup>i</sup>	BHD haplotype	NA	NA	25	17	1	10	6
240	NF <sup>i</sup>	BHD haplotype	NA	NA	17	8	2	4	1

NOTE.—ND = not determined or no CT scan available; NA = not applicable.

<sup>a</sup> Nucleotide numbering according to GenBank accession number AF517523. Nucleotide 456 corresponds to A of ATG initiation codon. In cases of ins/del in a mononucleotide tract, the first nucleotide of the tract is given.

<sup>b</sup> First codon affected by sequence variation. The codon for initiator methionine is codon 1.

<sup>c</sup> FS = frameshift (missense amino acids to predicted premature termination codon); SS = splice site; NS = nonsense.

<sup>d</sup> FF diagnosis based on histology, except where indicated.

<sup>e</sup> Diagnosis based on thoracic CT scan.

<sup>f</sup> PT = spontaneous pneumothorax episodes.

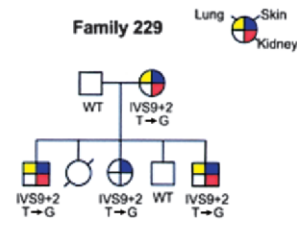
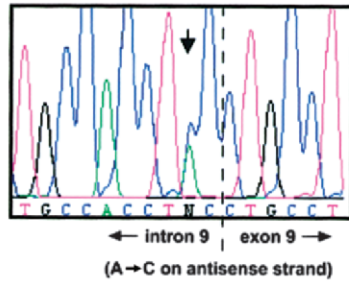
<sup>g</sup> FF diagnosis based on clinical evaluation only.

<sup>h</sup> Positive BHD diagnosis based on >80 trichodiscomas.

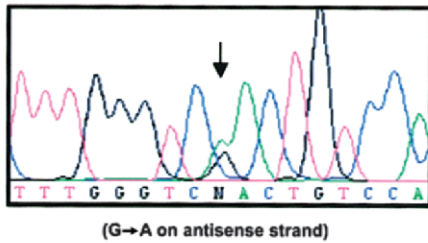
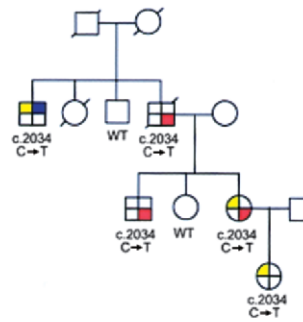
<sup>i</sup> NF = not found.

**A**

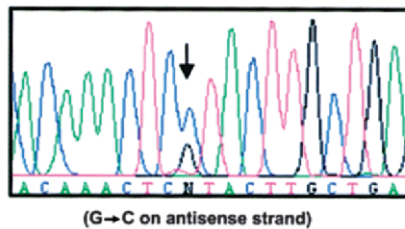
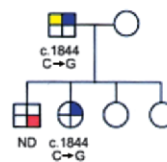
Mutation: IVS9+2T→G

**B**

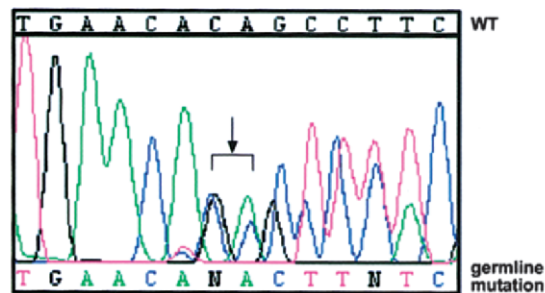
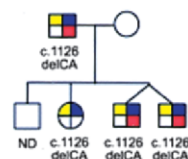
Mutation: c.2034C→T (R527X)

**Family 168****C**

Mutation: c.1844C→G (Y463X)

**Family 245****D**

Mutation: c.1126delCA (T224 frameshift)

**Family 166**

the 51 BHD-affected families (27 families had the hotspot mutation), exons 9 and 12 were each the site of mutations in 7 families, and exons 5 and 7 were each the site of mutations in 3 families. Exons 4, 6, 13, and 14 were each the site of one unique mutation in a BHD-affected family. Eight (16%) of the families had mutations in the 5' half, and 43 (84%) of the families had mutations in the 3' half of the *BHD* gene.

The most 5' mutation occurred at nt 513 (c.513delT) in exon 4 (20 aa from the initiation codon) in family 7306 (fig. 2). This frameshift mutation was predicted to truncate the protein 34 aa (missense) downstream. The affected individual in this family developed only BHD skin manifestations, without the other phenotypic features. The most 3' mutation occurred at nt 2034 (c.2034 C→T) in exon 14 (family 168) and was predicted to produce a premature termination codon at codon 527, eliminating only 52 aa from the full-length protein (fig. 1b). Nonetheless, full expression of the BHD phenotype was observed in family 168; three affected members developed renal neoplasia and lung cysts, and one was diagnosed with FF. Interestingly, the other nonsense mutations predicted to produce premature termination codons were located in exon 12 (c.1844C→G and c.1884C→T), also near the 3' end of the *BHD* gene.

Renal tumors were found in 5 (14%) of the mutation carriers with mutations in the 5' half of the *BHD* gene and in 30 (86%) of the mutation carriers with mutations in the 3' half of the *BHD* gene. Renal tumors were present in patients with frameshift mutations (insertion or deletion), nonsense mutations, and splice-site mutations in *BHD*. Of patients with spontaneous pneumothorax, 97% (55/57) inherited mutations in the 3' half of the *BHD* gene (table 1), and frameshift, nonsense, and splice-junction mutations were represented among those affected individuals.

#### Genotype-Phenotype Correlation: Mutational Hotspot

The most frequently mutated site in *BHD* was a cytosine insertion or deletion in the C<sub>8</sub> tract of exon 11 (c.1733insC or c.1733delC), identified in 27 BHD-affected families. We estimated the frequency of developing FFs, lung cysts, spontaneous pneumothorax, or renal tumors in BHD-affected family members with the C-insertion mutation compared with those individuals with the C-deletion mutation (fig. 3). A total of 64 individuals

carried the C-insertion mutation, and 27 individuals carried the C-deletion mutation. The frequency of histologically proven FFs among those individuals whose lesions were biopsied was similar among C-insertion-mutation carriers (83% [44/53]) and C-deletion-mutation carriers (87% [20/23]). The frequency of pneumothorax episodes was also similar between carriers of C-insertion (37% [23/62]) and C-deletion (35% [9/26]) mutations. Lung cysts were detected on thoracic CT scans in 88% (35/40) of C-insertion-mutation carriers, compared with 77% (13/17) of C-deletion-mutation carriers, which is not significantly different. However, we noted that renal tumors developed in significantly fewer C-deletion-mutation carriers than C-insertion-mutation carriers. Among C-deletion carriers, only 6% (1/16) of those who were evaluated by CT scan (4% [1/26] of those seen at the NIH or in the field) developed renal tumors. By comparison, 33% (13/40) of C-insertion-mutation carriers who were evaluated by CT scan (23% [13/56] of those seen at the NIH or in the field) developed renal tumors ( $P = .03$ ) (fig. 3). The median age (46.5–50 years) of patients with BHD seen at the NIH was similar for men and women with the C-insertion and the C-deletion mutations, and the number of males and females in each mutation group was approximately equal.

#### Genotype-Phenotype Correlation: Putative Splice-Site Mutation in IVS9

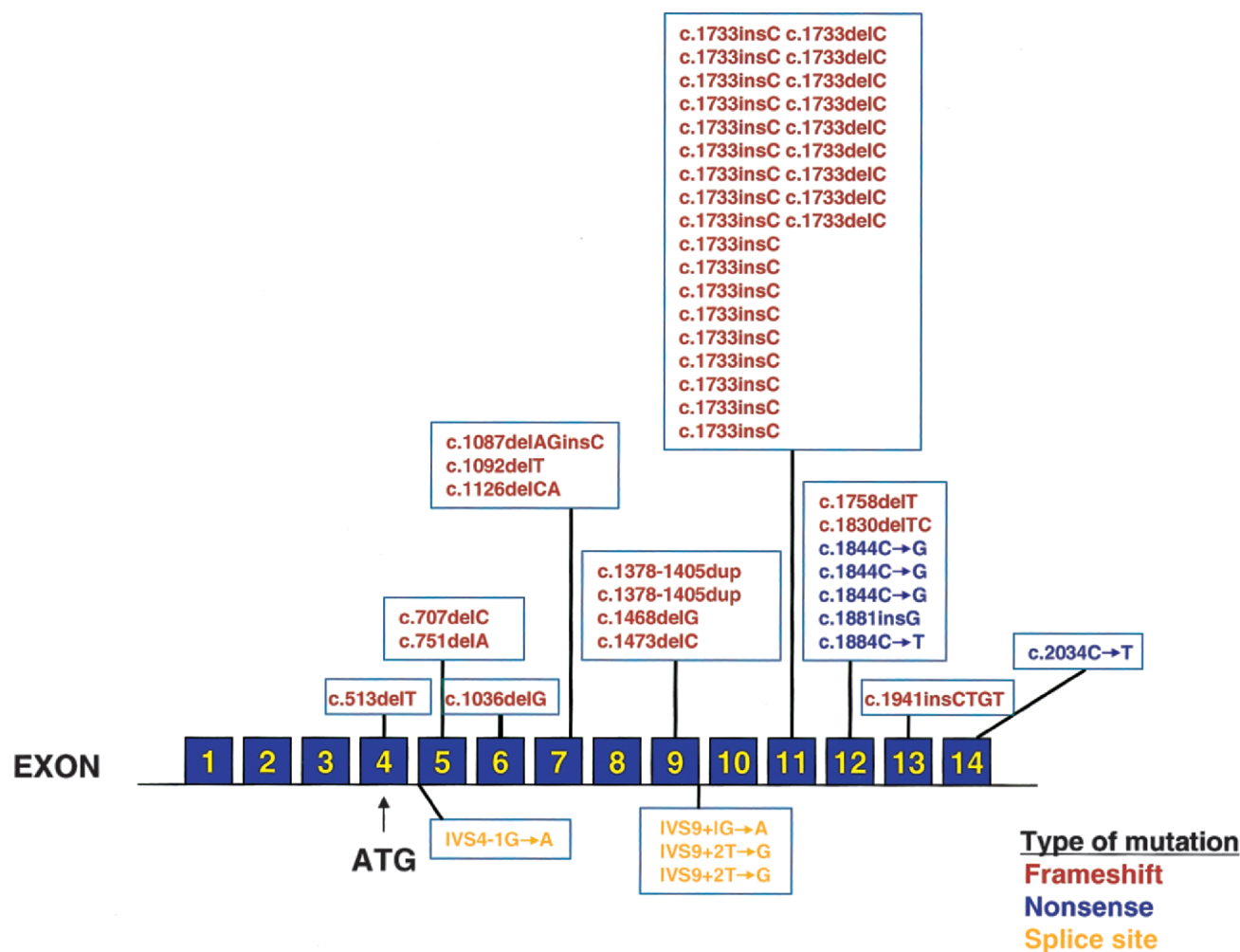
Two apparently unrelated families were found to have a putative splice-site mutation in the *BHD* gene within intron 9, IVS9+2T→G (table 1 and fig. 1a). Renal-tumor frequency in the affected members of families 229 and 238 was 3/5 and 3/3, respectively, which raises the possibility that this splice-site mutation might be associated with a higher frequency of renal tumors. A third family (family 242) inherited a different nucleotide change in the same splice-donor site (IVS9+1G→A) and had a renal-tumor frequency of 1/9 (table 1).

#### Linkage to 17p11.2 without Detectable BHD Mutation in Two Large Families with BHD

We screened 61 families with BHD that were affected with histologically proven FFs, and we found germline *BHD* mutations in 51 families. Two additional families, however, showed linkage to chromosome 17p11.2 by

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**Figure 1** *BHD* mutations in affected families. Sequencing chromatograms of genomic DNA of patients with BHD are shown on the left (the arrow indicates position of nucleotide variation), and family pedigrees are shown on the right. Quadrants within pedigree symbols indicate presence of FFs (blue), lung cysts/pneumothorax (yellow), and/or renal tumor (red) in each family member. WT = wild-type *BHD* sequence; ND = not determined; diagonal line = deceased. A, Mutation in a splice-donor site in intron 9 (IVS9+2T→G) is predicted to cause exon skipping. B and C, Mutations at c.2034C→T and c.1844C→G encode premature termination codons predicted to truncate the *BHD* protein. D, A 2-bp deletion, c.1126delCA, will cause a shift in the reading frame and lead to a premature stop codon downstream, truncating the *BHD* protein.



**Figure 2** *BHD* exon-intron structure showing the location of germline mutations identified in 51 families with BHD. Frameshift (red) and nonsense (blue) mutations, predicted to prematurely truncate the BHD protein, were identified along the entire length of the coding region (exons 4–14). Splice-site (yellow) mutations predicted to cause exon skipping were identified in introns 4 and 9. The insertion or deletion of a cytosine in a  $C_8$  tract in exon 11 was identified in 27 of 51 families with *BHD* mutations.

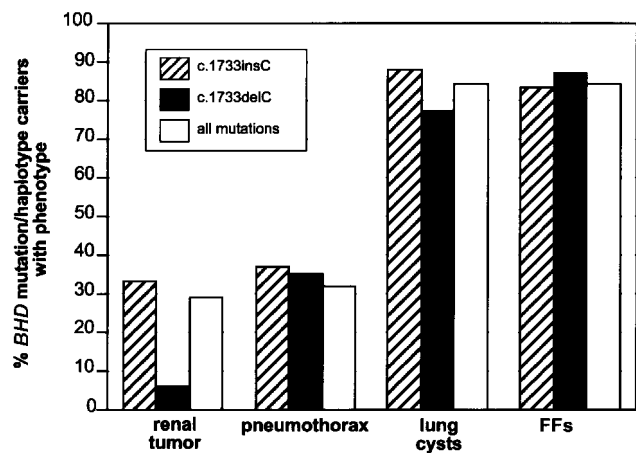
haplotype analysis with polymorphic microsatellite markers, but we were unable to detect a sequence variation in the *BHD* gene by direct sequence analysis. Evidence of heterozygosity at a number of informative SNP loci reduced the possibility of intragenic deletion in these families, although this deletion cannot be absolutely ruled out.

Family 172 is the original Canadian family described by Drs. Birt, Hogg, and Dubé (Birt et al. 1977) and was used in linkage analysis studies that identified the BHD-disease locus on 17p11.2 (LOD score 4.98 at marker *D17S740*;  $\theta = 0$ ) (Schmidt et al. 2001). Twenty-five family members had the same BHD-affected haplotype over an 8.5-cM region of chromosome 17p11-12 (Schmidt et al. 2001). However, direct sequencing of the 14 coding exons revealed no *BHD* sequence variation in periph-

eral-blood DNA from affected members of family 172. All family members whose lesions were biopsied had at least one histologically proven FF; one affected family member developed a renal tumor, and six members had a history of pneumothorax (table 1).

In family 240, a large family with BHD recruited to the NCI study, eight family members were identified with histologically proven FFs (Lindor et al. 2001). Upon genotyping with six polymorphic microsatellite markers flanking the *BHD* locus, from *D17S953* to *D17S1824*, 17 members were found to inherit the BHD-affected haplotype on chromosome 17p11.2 (data not shown). One *BHD*-haplotype carrier had a history of pneumothorax, two developed renal tumors, and four were found, on thoracic CT scan, to have lung cysts (table





**Figure 3** Frequency of BHD phenotypic manifestations in patients with the C-insertion mutation versus the C-deletion mutation in the  $C_8$  tract of exon 11. Patients with the c.1733delC mutation developed renal tumors at a significantly lower frequency than did patients with the c.1733insC mutation ( $P = .03$ ). Hatched bar indicates c.1733insC mutation carriers ( $n = 64$ ); blackened bar indicates c.1733delC mutation carriers ( $n = 27$ ); unblackened bar indicates all BHD-mutation/haplotype carriers ( $n = 219$ ), included for comparison. Renal tumors were identified from medical histories, surgical reports, and abdominal CT scans. Spontaneous pneumothorax episodes were determined by patient history and thoracic CT scans. Lung cysts were identified by thoracic CT scans. Skin biopsies were evaluated by a dermatologist for FF-positive histology.

1). Complete sequencing of *BHD* in DNA from affected-haplotype carriers in family 240 did not reveal any sequence variation.

## Discussion

In the present study, we completed *BHD*-mutation analysis, by direct sequencing, of 30 families with BHD that were recruited to the NCI. To date, we have identified mutations in 84% (51/61) of all families with BHD who were studied at the NCI, including 16 novel insertion/deletion mutations, 3 nonsense mutations, and 3 putative splice-site mutations. The majority of these mutations were predicted to prematurely terminate folliculin and to result in loss of function, suggesting that *BHD* may act as a tumor-suppressor gene.

These data support other lines of evidence that point to a tumor-suppressor role for *BHD*. The majority of germline *BHD* mutations reported to date are inactivating frameshift and nonsense mutations (Khoo et al. 2002; Nickerson et al. 2002; the present study). Loss of the wild-type *BHD* allele (loss of heterozygosity [LOH] 17%) or somatic frameshift second-hit mutations (53%) were confirmed in 77 BHD-associated renal tumors from kindreds with BHD (Vocke et al., in press),

resulting in biallelic inactivation of *BHD*. A novel somatic mutation was identified in a BHD-related chromophobe RCC from an affected member of a family with BHD (Khoo et al. 2002). Additionally, two animal studies of BHD have been described that support a tumor-suppressor function for the BHD protein, the Nihon rat renal cancer model (Okimoto et al. 2000, 2004; Hino et al. 2001) and canine hereditary renal cystadenocarcinoma and nodular dermatofibrosis in the German shepherd (Lium and Moe 1985; Jonasdottir et al. 2000; Lingaas et al. 2003). Germline disease-co-segregating mutations have been identified in the *Bhd* rat and dog orthologs that lead to renal-tumor development in these animal models of BHD. The germline frameshift mutation in the Nihon rat *Bhd* ortholog is predicted to prematurely truncate the encoded protein.

We noted that, among the 53 BHD-affected families, no disease-associated missense mutations in the *BHD* gene were found. One possible explanation is that missense *BHD* mutations that lead to amino acid substitutions may have little or no effect on folliculin function, thereby producing either a mild (undetectable) phenotype or no phenotype at all.

No correlation between BHD phenotype and location of the mutation was found. We found disease-associated mutations along the entire length of the *BHD* gene, within 9 of the 11 coding exons. The location of the mutation did not affect the phenotypic expression of BHD. Renal tumors, lung cysts, spontaneous pneumothorax, and FFs developed in patients with mutations in both the 5' half and the 3' half of the gene. Since these mutations were predicted to introduce a premature termination codon, it is most likely that the mutant mRNA transcribed from the genomic DNA of these patients is degraded by nonsense-mediated decay (NMD) (Maquat 2004), a surveillance mechanism that eliminates mRNAs with premature termination codons in all but the last exon of a gene. If NMD effectively eliminates the *BHD* mutant mRNAs, then phenotypic variation among *BHD*-mutation carriers may reflect the efficiency of inactivation of the *BHD* wild-type allele by LOH or somatic mutation in tissues involved in the BHD phenotype. Additionally, genetic or environmental factors may contribute to the variability of BHD phenotypic expression. In support of this idea, the BHD phenotype in patients varies within and between families who harbor the same *BHD* germline mutation but who most likely have different genetic backgrounds and/or environmental exposures.

In an effort to identify genotype-phenotype correlations, we took advantage of the large number of individuals with the c.1733ins/delC mutation in *BHD* and compared the phenotype of patients who harbored the C insertion with patients who inherited the C deletion.

Interestingly, the frequency of renal tumors in C-insertion carriers was significantly greater than the frequency of renal tumors in C-deletion carriers. If mutant *BHD* mRNA were not completely degraded by NMD but were at least partially translated to mutant folliculin, the C-insertion and C-deletion mutations would produce very different aberrant BHD proteins. One could speculate that the residual mutant protein produced in patients with the C-insertion mutation might have a dominant-negative effect and interfere with the wild-type BHD protein function, leading to the development of renal tumors. Alternatively, the residual mutant protein encoded by the C-insertion mutation might predispose kidney cells to form a renal tumor by some as-yet-unknown mechanism.

Among the BHD-affected family members with putative splice-site mutations in intron 9 predicted to cause exon skipping, 7 (40%) of 17 developed renal tumors, a significantly higher frequency than the overall frequency of renal tumors seen in all mutation carriers. It is possible that the aberrant BHD protein produced by exon skipping might predispose to the development of renal tumors by interacting with the wild-type BHD protein and compromising its function. The identification of the incorrectly spliced mutant *BHD* gene product and the study of its functional properties will contribute to our understanding of how mutations in *BHD* lead to renal-tumor development.

Analysis of the *BHD* gene identified mutations in 51 of 53 NCI-study families with BHD who inherited the BHD-affected haplotype; however, we were unable to identify mutations in two of those families by direct sequencing. The BHD-affected haplotypes, which cosegregated with disease in families 172 and 240, were tightly linked to the *BHD* locus on chromosome 17p11.2, and the full BHD phenotype was seen in affected individuals from these two families. Although the entire coding sequence for *BHD* was screened, the 5' and 3' regulatory regions and intronic sequences of the gene were not examined for sequence variation that might affect *BHD* expression, and the possibility of a small intragenic deletion cannot be ruled out. Careful evaluation of *BHD* for deletions or sequence variants in regulatory regions of the gene in families 172 and 240 is in progress.

In conclusion, we have completed mutation analysis of the largest cohort of families with BHD reported to date and have identified 22 different germline *BHD* mutations in 84% (51/61) of families recruited to our study. The majority of mutations were predicted to truncate folliculin and most likely lead to a reduction in folliculin expression. Renal tumors, lung cysts, spontaneous pneumothorax, and FFs develop as part of the complex BHD phenotype, with no particular correlation with mutation type or location within the gene.

A mutational hotspot, a C insertion/deletion in exon

11, is frequently mutated in BHD-affected families. Renal tumors developed with a higher frequency in C-insertion carriers than in C-deletion carriers and also more frequently in individuals who inherited a putative splice-site mutation in intron 9. The mechanism by which *BHD* acts as a tumor suppressor and its role in the development of renal tumors, benign skin hamartomas, and lung-wall defects remains to be elucidated, which may not be possible until the BHD protein is characterized. BHD should be considered in the differential diagnosis for patients with renal tumors with or without skin papules and for patients with a family history of spontaneous pneumothorax.

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## Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

Calculation of Fisher's Exact Test, <http://www.unc.edu/~preacher/fisher/fisher.htm>  
 GenBank, <http://www.ncbi.nlm.nih.gov/Genbank/> (for *FLCN*, or *BHD* [accession number AF517523; Ref Seq NM\_144997], skin melanoma [BC015725 and BC015687], and BAC clone RP11-45M22 [accession number AC055811])  
 Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for BHD and *FLCN*)

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