Mutations in a novel gene lead to kidney tumors, lung wall defects, and benign tumors of the hair follicle in patients with the Birt-Hogg-Dubé syndrome

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Summary

Birt-Hogg-Dubé (BHD) syndrome is a rare inherited genodermatosis characterized by hair follicle hamartomas, kidney tumors, and spontaneous pneumothorax. Recombination mapping in BHD families delineated the susceptibility locus to 700 kb on chromosome 17p11.2. Protein-truncating mutations were identified in a novel candidate gene in a panel of BHD families, with a 44% frequency of insertion/deletion mutations within a hypermutable C8 tract. Tissue expression of the 3.8 kb transcript was widespread, including kidney, lung, and skin. The full-length BHD sequence predicted a novel protein, folliculin, that was highly conserved across species. Discovery of disease-causing mutations in BHD, a novel kidney cancer gene associated with renal oncocytema or chromophobe renal cancer, will contribute to understanding the role of folliculin in pathways common to skin, lung, and kidney development.

Introduction

Kidney cancer is a major health problem; worldwide, about 150,000 people develop kidney cancer, with 78,000 deaths from metastatic disease each year because available treatments are ineffective (Zbar et al., 2002b). Mutation screening of inherited kidney cancer genes in affected families allows presymptomatic diagnosis of family members who are at risk, providing better patient prognosis and disease management.

Kidney cancer may be classified into 4 histologic types: (1) clear cell (CCRC, 75%), (2) papillary (PRC, 15%), (3) oncocytema (5%), and (4) chromophobe (5%) (Kovacs et al., 1997). CCRC develops in patients with inherited von Hippel-Lindau (VHL) disease, a multisystem neoplastic disorder. Mutations in the VHL tumor suppressor gene are found in the germline of VHL patients and in 50% of sporadic CCRC (Latif et al., 1993). The VHL gene product, pVHL, is involved in the control of hypoxia-inducible genes by targeting hypoxia-inducible factor-1α (HIF-1α) for ubiquitin-mediated degradation. Mutational inactivation of VHL leads to the development of highly vascular tumors through...
The BAC tiling path is shown by black horizontal lines with arrowheads indicating directional read of completed sequence and Genbank accession numbers. BAC overlaps were confirmed by in silico and PCR methods. A single gap was spanned by exons of the COPS3 gene. Locations of polymorphic markers, shown in red, and genes, shown in blue, were confirmed in silico and by PCR amplification from BAC clones. Telomere; centromere.

B: Critical recombinants identified in Family 243 (D17S2196), Family 210 (CA109), and Family 216 (CA138) define the BHD minimal region to 700 kb. Nonrecombining region is in green.

C: Location of 2 overlapping, uncharacterized mRNAs from melanoma (Genbank accession numbers BC015725 and BC015687) are shown within the 700 kb BHD candidate region. The BHD gene exon/intron structure with 14 coding exons is given.

upregulation of hypoxia-inducible genes (Kondo and Kaelin, 2001).

Hereditary papillary renal carcinoma (HPRC) occurs less frequently than CCRC and predisposes patients to develop bilateral, multifocal papillary renal tumors (Zbar et al., 1995). Activating mutations in the tyrosine kinase domain of the MET proto-oncogene were found in HPRC Type I (Schmidt et al., 1997, 1999), which constitutively phosphorylate and activate the MET protein (Jeffers et al., 1997). HPRC Type 2 is associated with inactivating mutations in the fumarate hydratase gene, a Krebs cycle enzyme, which cause hereditary leiomyoma/renal cell carcinoma (HLRCC) and multiple cutaneous leiomyoma (MCL) (TMLC, 2002).

The cancer-causing genes responsible for renal oncocyto ma and chromophobe renal carcinoma have not yet been identified. To identify genes involved in the pathogenesis of renal oncocyto ma, 5 families with multiple, bilateral renal oncocyto mas were identified and clinically evaluated (Weirich et al., 1998). Interestingly, after noting multiple skin papules (fibrofolliculomas) on the face and neck of a pair of identical twins with bilateral renal oncocyto mas, reevaluation of the oncocyto ma families revealed a diagnosis of Birt-Hogg-Dubé syndrome (BHD) in 3 of 5 families (Toro et al., 1999).

We considered whether BHD skin lesions (fibrofolliculomas) could be used as a marker to identify a novel kidney cancer gene associated with renal oncocyto ma. Birt-Hogg-Dubé syndrome (BHD) was originally described in a Canadian kindred in 1977 (Birt et al., 1977). Fibrofolliculomas appear as white or skin-colored papules on the face and upper torso. Histologically, they are characterized as multiple, anastomosing strands of proliferating epithelial cells extending from a central hair follicle. Other phenotypic features found to be associated with BHD included renal neoplasia (Roth et al., 1993; Pavlovich, et al., 2002), lung cysts, and/or spontaneous pneumothorax (Toro et al., 1999; Binet et al., 1986). Patients with fibrofolliculomas have an increased risk for developing renal neoplasms (7-fold) and spontaneous pneumothorax (50-fold) compared with their unaffected siblings (Zbar et al., 2002a). In a study of 130 renal tumors found in 30 BHD patients, the spectrum of renal histology included 34% chromophobe, 5% oncocyto ma, 50% chromophobe/oncocytic hybrid, 9% clear cell, and 2% papillary (Pavlovich, et al., 2002).
Table 1. BHD gene mutations in a panel of 9 families with BHD syndrome

<table>
<thead>
<tr>
<th>Family</th>
<th>Exon</th>
<th>Mutation</th>
<th>Predicted result</th>
</tr>
</thead>
<tbody>
<tr>
<td>202</td>
<td>7</td>
<td>1087delAGinsC</td>
<td>frameshift, protein truncation</td>
</tr>
<tr>
<td>228</td>
<td>9</td>
<td>1378→1405dup</td>
<td>frameshift, protein truncation</td>
</tr>
<tr>
<td>174</td>
<td>11</td>
<td>1733insC</td>
<td>frameshift, protein truncation</td>
</tr>
<tr>
<td>200</td>
<td>11</td>
<td>1733insC</td>
<td>frameshift, protein truncation</td>
</tr>
<tr>
<td>210</td>
<td>11</td>
<td>1733insC</td>
<td>frameshift, protein truncation</td>
</tr>
<tr>
<td>216</td>
<td>11</td>
<td>1733delC</td>
<td>frameshift, protein truncation</td>
</tr>
<tr>
<td>201</td>
<td>11</td>
<td>1733delC</td>
<td>frameshift, protein truncation</td>
</tr>
<tr>
<td>230</td>
<td>12</td>
<td>C1844G</td>
<td>Tyr463X</td>
</tr>
</tbody>
</table>

*a* Mutations are named according to recommendations of the Nomenclature System for Human Gene Mutations. The GenBank mRNA sequence (AF517523) of BHD is used for reference. The A of the ATG of the initiator codon is denoted as nt 456. An additional 14 of 53 families had the 1733insC and 8 of 53 families had the 1733delC.

We pursued the identification of renal oncocytoma and chromophobe renal carcinoma genes by taking advantage of the phenotypic association of BHD with these histologic types of renal cancer, and recruited families on the basis of a diagnosis of BHD. We performed linkage analysis in 9 BHD families and localized the BHD disease locus to a 4 cM region of chromosome 17p11.2 between D17S1857 and D17S805 (Schmidt et al., 2001), which was confirmed in a Swedish BHD pedigree with associated renal neoplasms (Khoo et al., 2001). In this report, we have used recombination mapping to narrow the BHD disease locus and identified disease-associated mutations in a novel gene which predisposes patients to develop oncocytic and chromophobe renal tumors, collapsed lung/lung cysts, and BHD skin lesions.

**Results**

**Physical map of BHD critical region spans 1.5 Mb and defines BHD candidate genes for analysis**

Our previous studies defined the BHD gene locus to a 4 cM region of chromosome 17p11.2 between D17S1857 and D17S805 by linkage analysis in 9 BHD families. As an initial step in the positional cloning of the BHD gene, we established a physical map of the region of nonrecombination derived from available genomic sequence produced by the large-scale public sequencing laboratories and Celera. We refined the BAC tiling path map by in silico methods using BLAST (Altschul et al., 1990) and comparative analysis of genome assemblies (UCSC Human Genome Browser, Celera, NCBI, and Ensembl), and identified known genes, uncharacterized mRNAs, and spliced EST clusters in the 17p11.2 critical region (Figure 1A). A PCR-based approach was used to amplify microsatellites and coding exons of genes to confirm their locations on overlapping BACs. These results and fluorescence in situ hybridization data (V.M., unpublished data) provided additional support for the BAC order. This 1.5 Mb BAC physical map is in agreement with the physical map of Lucas et al. (2001), but disagrees with the current UCSC Genome Browser (December 2001) and Celera and NCBI (April 2002) genome assemblies.

**High-throughput BHD candidate gene mutation analysis**

is used to evaluate a total of 39 genes, mRNAs, and ESTs on 17p11.2

We identified candidate genes from the critical region based on expressed sequence tag (EST) evidence of expression in skin, lung, and/or kidney. We determined the exon/intron gene structure, designed intronic primers to amplify coding sequences and splice...
mutations, and performed high-throughput mutation analysis on a panel of patient DNA samples, representing 9 BHD families. Prior to the identification of the BHD gene (see below), we sequenced 321 coding amplicons representing 39 known genes, uncharacterized mRNAs, and spliced EST clusters from the 4 cM region of linkage. This effort identified 129 coding SNPs (single nucleotide polymorphisms), 49 intronic SNPs, 7 polymorphic repeats (i.e., poly A tracts), and 6 insertions/deletions. Since no mutations were identified among these amplicons, we pursued more detailed recombination mapping.

Additional critical recombinants narrow the BHD candidate gene region to 700 kb
In parallel with BHD candidate gene sequencing, we developed 13 polymorphic microsatellite markers to look for additional recombinants in the region of linkage. Further genotyping and haplotype analysis of BHD Family 210, described previously (Schmidt et al., 2001), identified a recombination in the distal marker CA109, eliminating genes that were telomeric of CA109 from consideration as candidate genes. We analyzed additional BHD families by genotyping and haplotype analysis and identified a proximal recombination in BHD Family 216, in the marker CA138, which localized the BHD gene to a 1.3 Mb region between CA109 and CA138 (Figure 1A). Subsequently, we identified a proximal recombination in BHD Family 243, at D17S2196, which narrowed the BHD critical region further to 700 kb (Figure 1B).

Tissue expression of two overlapping mRNAs from the BHD critical region suggests a single, potential BHD candidate gene
Gene mining within the 700 kb critical region using the UCSC Human Genome Browser (December 2001) identified two overlapping, uncharacterized, full-length transcripts from skin melanoma (BC015725 and BC015687). The coding sequences were supported by additional lung, kidney, and melanoma ESTs (Figure 1C). Since BC015725 and BC015687 mRNAs (1.9 kb and 1.7 kb, respectively) shared an overlapping coding exon, we considered the possibility that they represented alternatively spliced variants.
of a single gene. We performed Northern blot analysis, using hybridization probes designed from each mRNA, to determine if they coded for the same or different transcripts. Hybridization with probes from either mRNA revealed a 3.8 kb transcript in most normal adult tissues, including skin, lung, and kidney (Figure 4A), and in fetal lung, kidney, liver, and brain tissue (data not shown). The 3.8 kb mRNA transcript size was roughly equal to the sum of BC015725 and BC015687 mRNAs, suggesting that these two transcripts represent splice variants of a single mRNA that is widely expressed. We regarded the novel gene represented by BC015725 and BC015687 mRNAs as an excellent BHD candidate gene based on the source, tissue expression pattern of the two overlapping mRNAs, and their location in the 700 kb BHD critical region.

**Mutation analysis of the putative BHD gene reveals frameshift mutations which are predicted to truncate the protein**

The exon/intron structure of the BHD candidate gene represented by BC015725 and BC015687 was determined, and intronic primers were designed to amplify coding sequences and splice junctions from a panel of 9 BHD family probands and 3 unaffected family members. Sequence analysis of the 14 coding exons contained in these two mRNAs revealed frameshift or termination mutations in 8 of 9 BHD families on the panel (Table 1).

A cytotoxic insertion mutation in a mononucleotide (C)_n tract (nt 1733–1740) in exon 11 was identified in BHD Families 174, 200, 210, and 216, resulting in a frameshift predicted to truncate the protein 26 missense amino acids downstream. A cytotoxic deletion mutation in the same mononucleotide (C)_n tract was identified in Family 201 which would truncate the protein 38 missense amino acids downstream. In order to separate the mutant C_n (or C_n) allele from the wild-type C_n allele for sequence analysis, each allele was isolated in a somatic cell hybrid (Papadopoulos et al., 1995). Sequence analysis of the somatic cell hybrids established from patients from several of these BHD families confirmed the presence of the (C)_n allele on the affected chromosome 17 and the (C)_n allele on the wild-type chromosome 17. Cosegregation of these C tract insertion/deletion mutations in BHD-affected haplotype carriers was confirmed by sequencing (Figure 2A).

A complex mutation, delAGinsC, which resulted in a frame shift and predicted protein truncation 11 missense amino acids downstream, was identified in Family 202 in exon 7 at nt 1087–1088. This complex mutation was shown to cosegregate with disease by DHPLC (Figure 2B). The unique DHPLC 2-peak chromatographic profile was produced by separation of heteroduplexes, formed between wild-type and mutant allele amplicons, to truncate the protein 26 missense amino acids downstream. A fourth mutation was identified in BHD Family 230, a C to G at nt 1844, which produced an in-frame termination at codon 463, resulting in a frameshift predicted to truncate the protein 26 missense amino acids downstream. A cytosine insertion mutation in a mononucleotide (C)_n tract in exon 12 (Figure 2C). Each family’s mutation was present in affected members and asymptomatic BHD haplotype carriers.
within that family, but was absent in noncarriers and at least 160 normal individuals.

**Hypermutable mononucleotide C tract in exon 11 is mutated in 44% of BHD families tested**

We screened an additional 53 probands from small BHD families for mutations in the mononucleotide (C)n tract in exon 11 of the BHD gene. We found C insertions or deletions in 22 of the 53 probands, suggesting that this cytosine mononucleotide tract is hypermutable and particularly prone to disease-causing mutations (Table 1). We have identified a total of 18 (C)n mutations and 9 (C)n mutations in 62 BHD patient samples, a (C)n tract mutation frequency of 44%. Insertion/deletion mutations in genes with homonucleotide tracts have been reported in other inherited human disorders (Rodenhiser et al., 1996, 1997). A slippage-mediated mechanism during DNA replication of single base repeats may result in these frameshift mutations leading to protein truncation (Streisinger et al., 1966). A founder effect/ancestral mutation in this rare disease is an alternative possibility to hypermutability.

**Full length BHD sequence predicts a novel protein, folliculin, conserved across species**

cDNAs from adult kidney and adult and fetal lung (Clontech) were used to amplify 3.2 kb of the BHD transcript and sequenced to >4-fold coverage. Separately, a putative full-length clone was obtained by screening a normal lung cDNA library (Origene Technologies, Inc.) and was also sequenced to >4-fold coverage. The full-length BHD sequence of 3674 nucleotides (AF517523) predicted a protein, folliculin (named for the BHD skin lesion, fibrofolliculoma), with an open reading frame of 579 amino acids (Figure 4B). Programs which included in SEQWEB and PROSITE predicted a 64 kDa cytoplasmic protein with a glutamic acid-rich, coil-coil domain, one N-glycosylation site, three myristoylation sites, and several casein kinase II and protein kinase C phosphorylation sites. Although unusual, the presence of a glutamic acid-rich, coil-coil domain suggests a function for folliculin as a component of the cytoskeletal network. The histology of the stroma-rich skin lesions suggests a defect in epithelial-mesenchymal interaction.

Folliculin is a novel protein. Sequence conservation has been found in the mouse, *D. melanogaster*, and *C. elegans*, underscoring an important biologic function for the BHD gene in a variety of organisms. Additional support for the importance of the BHD gene comes from kidney cancer models in the dog and the rat. Germline mutations in BHD orthologs which map to disease-associated, syntenic locations in the dog and rat may be responsible for naturally occurring inherited renal malignancies in these species (Jónasdóttir et al., 2000; Hino et al., 2001).

Genes have been identified that cause two of the four main histologic types of renal cancer: *VHL*, responsible for clear cell renal carcinoma; *MET*, responsible for papillary renal carcinoma Type I; and *FH*, responsible for papillary renal carcinoma Type 2. This report describes the discovery of the a novel gene with disease-associated mutations that predispose to the development of kidney tumors with oncocytoma and chromophobe histologies. How do germline mutations of the BHD gene lead to renal tumors? The consistent finding of truncating mutations in the BHD gene suggests that the BHD gene might be a tumor suppressor gene. Loss of heterozygosity studies should address this possibility. Alternatively, renal tumors may develop by a haploinsufficiency mechanism or as a consequence of hypermethylation of the wild-type BHD allele. Another possibility may be that the inactive BHD allele produced by germline mutations results in a dominant-negative effect leading to the BHD phenotype.

Clues to the mechanism of renal tumorigenesis in the BHD syndrome may come from consideration of the disease phenotype. The lung cysts and lung collapse (pneumothorax) that are features of BHD suggest a defect in a structural protein of the lung wall, which may be a component of the cytoskeletal network. The histology of the stroma-rich skin lesions suggests a defect in epithelial-mesenchymal interaction.

Renal tumor development in patients affected with BHD is unusual. In contrast to other forms of inherited renal cancer in which affected individuals develop multiple, bilateral renal tumors of a specific histologic type, individuals affected with BHD have a predisposition to develop different histologic types of renal cancer, including oncocytoma, chromophobe, and clear cell. Clear cell renal carcinomas are thought to originate from the proximal renal tubule, while renal oncocytomas and chromophobe renal carcinomas are thought to originate from the distal renal tubule. All of these histologic types may develop within a single kidney of a BHD-affected individual, suggesting that the BHD mutation affects the progenitor cell for both distal and proximal renal tubules. Perhaps the mutation alters the composition of the extracellular matrix, producing a structural or microenvironmental abnormality that affects the cells of both the proximal and distal renal tubules, leading to uncontrolled cellular proliferation. We anticipate discovery of novel mechanisms of tumorigenesis through study of this unusual inherited disorder.

**Discussion**

Using a classic positional cloning strategy, we have identified a gene that plays a critical role in the pathogenesis of an uncommon form of kidney cancer through its association with the more common and clinically detectable phenotype of an inherited disorder of the hair follicle. All mutations discovered in the germline of affected members of BHD families were predicted to truncate the protein, suggesting a loss-of-function mechanism for phenotype development.

Inspection of the predicted amino acid sequence of the BHD protein, folliculin, and BLAST alignment with available protein databases has not disclosed homology to any known gene or gene family, or identified key functional domains. This suggests that
designated to amplify potential polymorphic microsatellites, and markers were selected with a heterozygosity >0.6 in a panel of eight unrelated individuals. Primers and PCR conditions are available upon request. Microsatellite genotyping and haplotype analysis were performed as described (Schmidt et al., 2001).

Candidate gene selection and analysis
The BHD critical region at 17p11.2 was examined for known genes, uncharacterized mRNAs, and EST clusters using the UCSC Human Genome Browser as a primary reference, with details from Celera, NCBI, and Ensembl human and mouse genome assemblies, and BAC clone annotation by Doubletwist.

Methods for identification of exon/intron boundaries, selection of primers, PCR conditions, and high-throughput DNA sequencing and analysis can be found in the Supplemental Experimental Procedures (see Supplemental Data, below).

Analysis of the BHD gene
Two overlapping, uncharacterized, full-length transcripts from melanoma (MGc project; BC015725 and BC015687), in the December 2001 UCSC Genome Browser release, highlighted a spliced EST cluster on BAC clone RP11-45M22 (AC055811), which was analyzed for mutations. Details of BHD gene analysis are given in the Supplemental Experimental Procedures (see Supplemental Data).

Northern blot analysis
Expression of the BHD gene transcript was evaluated with human poly A+ RNA tissue blots (Origene Technologies, Inc.) and a human poly A+ RNA fetal blot (Clonetech). Details of Northern analysis are described in the Supplemental Experimental Procedures (see Supplemental Data).

Somatic cell hybrid cell lines
Lymphoblasts from several BHD patients (2×10^7) were fused with mouse RAG cells (2×10^7 cells; HPRT-deficient mouse cell line CCL-142 from American Type Culture Collection) with HAT selection. Methods are detailed in the Supplemental Experimental Procedures (see Supplemental Data). DNA was prepared from expanded colonies and genotyped to determine whether one copy or both copies of human chromosome 17 were present.

Isolation and sequencing of full length BHD cDNA clones
cDNA was obtained from normal adult kidney, and adult and fetal lung (Clonetech). Gene-specific primers were designed to amplify BHD cDNA. Independently, Origene Technologies, Inc. screened several cDNA libraries and isolated a longest clone from lung. Clones were shotgun sequenced; the details of the methods are provided in the Supplemental Experimental Procedures (see Supplemental Data).

Supplemental data
Supplemental Experimental Procedures, URLs, and Table S1 containing BHD primer sequences and PCR conditions are available in the Supplemental Data at http://www.cancercell.org/cgi/content/full/2/2/157/DC1.

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Accession numbers

The full-length BHD sequence has been deposited in the GenBank under accession number AF517523.