ELECTRONIC LETTER

# Exclusion of *PTEN*, *CTNNB1*, and *PTCH* as candidate genes for Birt-Hogg-Dube syndrome

**J R Toro, Y O Shevchenko, J G Compton, S J Bale**

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In 1977, Birt, Hogg, and Dube described a kindred in which 15 of 70 members over three generations exhibited multiple, small, grey skin coloured, dome shaped papules distributed over the face, neck, and upper trunk inherited in an autosomal dominant pattern.[1] Histological examination of these lesions showed fibrofolliculomas, trichodiscomas, and acrochordons. This triad has become known as Birt-Hogg-Dube (BHD) syndrome. Since the initial report, other cases have been described.[2–13]

The cutaneous manifestations of BHD, which typically appear during the third or fourth decade of life, have been associated with renal carcinoma,[2 5] spontaneous pneumothorax,[2 6] and colonic polyps.[3 4] Toro *et al*[2] recently reported three extended kindreds in whom renal neoplasms and BHD segregated together. Two kindreds had renal oncocytomas and a third had a variant of papillary renal cell carcinoma. Renal neoplasms can be familial or sporadic. Four types of familial renal neoplasms have been well described: (1) clear cell renal carcinoma associated with haemangioblastomas of the brain, spine, and eye owing to mutations in the von Hippel-Lindau (VHL) disease tumour suppressor gene,[4] (2) clear cell renal carcinoma associated with constitutional, balanced translocations involving the short arm of chromosome 3,[15–17] (3) papillary renal cell carcinoma associated with germline mutations in the tyrosine kinase domain of the *MET* proto-oncogene,[18] and (4) renal oncocytoma.[19]

In this study, we report an extended family that we evaluated for *PTEN*, *CTNNB1*, and *PTCH* as candidate genes for BHD and renal cancer. These genes were selected for study because mutations in each are associated with a disorder that has clinical features that overlap BHD and because each of these disorders carries an increased risk for internal malignancy. We used a candidate gene approach. We first performed sequencing analysis, but no mutations were identified. Then, to exclude mutations in the promoter, we decided to do genetic linkage analysis.

The gene mutated in many patients with Cowden syndrome, *PTEN*, has been mapped to 10q23.3. Cowden syndrome shares many phenotypic features with BHD, such as follicular hamartomas, mucosal fibromas, and internal malignancy.[20 21]

*PTCH*, the gene for basal cell naevus syndrome, is located at 9q22.3. Basal cell naevus syndrome shares with BHD the clinical presentation of multiple facial follicular papules and also histologically fibrofolliculoma and basal cell carcinoma share the presence of palisading of cells in myxoid or fibrotic stroma. Both BHD and basal cell naevus syndrome also have an increased risk for internal malignancy.[22 23] *CTNNB1,* the gene for β-catenin, is located at 3p21. Mutations in β-catenin have been reported in most pilometricomas.[24 25] Fibrofolliculomas and pilometricomas are benign tumours derived from the hair follicle. In addition, *CTNNB1* was a good candidate gene because genes in the WNT pathway have been found to play an important role in kidney embryogenesis and mutations in *CTNNB1* have been reported in some renal tumours.[26 27]

## PATIENTS AND METHODS

Twenty-eight subjects from a large kindred were enrolled in an Institutional Review Board approved study at the National Institute of Health between June 1998 and May 2000. All patients underwent a complete skin and oral examination and were photographed. Some patients were evaluated by pre- and post-contrast enhanced computed tomography (CT) of the abdomen followed by renal ultrasound as previously described.[2] Solid lesions seen on CT scan and ultrasound were taken as evidence of renal tumours. Lesions were considered indeterminate if they were too small (2-5 mm) to be classified as either cyst or solid. Subjects without renal tumours by CT examination were classified as unaffected with kidney tumours. In addition, the diagnoses of renal cell carcinoma, fibrofolliculoma, and colonic polyps were also based on the review of death certificates, medical records, and pathology and necropsy reports. Nineteen subjects had colonoscopy to the ileocecal valve followed by polypectomy of suspicious lesions. Mucocutaneous lesions were diagnosed clinically. The diagnosis of BHD was confirmed by histological examination of lesional skin biopsy specimens. Histologically, fibrofolliculomas were defined as multiple anastomosing strands of epithelial cells extending from a central follicle into the dermis.[1] The diagnosis of BHD was made in patients who had five or more facial or truncal papules of which at least one was confirmed histologically as a fibrofolliculoma.

DNA for analysis was prepared from buccal swabs collected from consenting patients and processed.[28] Sequence specific PCR primers were designed based on published sequences, with Tms adjusted to approximately 60°C. The sequences M13F (CACGACGTTGTAAAACGAC) and M13R (GGATAACAATTTCACACAGG) were added to the 5′ ends of forward and reverse primers, respectively; 2 μl of each sample was used as template in each PCR assay. DNA fragments were amplified (DNA Engine, MJ Research, Watertown, MA, USA) using 2 μl of buccal sample in 25 μl volume with 1.25 μl of Red*Taq* DNA Polymerase (Sigma, St Louis, MO, USA) and 10 pmol of each primer using the temperature profile: 94°C for two minutes, then 94°C for 15 seconds, 60°C for 30 seconds, 72°C for one minute repeated 35 times. PCR reactions were loaded on a 2% agarose gel (Seakem LE, FMC, Rockland, ME, USA) in 1 × TAE buffer. Fragments of the expected length were cut out, purified using the Ultra Clean GelSpin purification kit (MoBio, Solana Beach, CA, USA) according to the manufacturer's protocol, and recovered in 50 μl elution.

### Bidirectional dideoxy fingerprinting for *PTCH*

Bidirectional dideoxy fingerprinting was done as described previously.[29]

**Abbreviations:** BHD, Birt-Hogg-Dube syndrome; BCNS, basal cell naevus syndrome; RCC, renal cell carcinoma; RCND, renal cystadenocarcinoma and nodular dermatofibrosis; TSC, tuberous sclerosis complex

**Table 1** Dermatological, renal, gastrointestinal, and molecular findings in patients with Birt-Hogg-Dube syndrome and at risk relatives

| | | Dermatologic findings | | | | | | | Molecular studies | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | FF/TD | ST | OP | L | Renal findings | Colonoscopy | BHD | *PTEN* | *PTCH* | *CTNNB1* |
| I:1 | 80/F | + | NA | NA | NA | No studies or history | Not done | + | ND | ND | ND |
| I:2 | 80/F | + | NA | NA | NA | No studies or history | Not done | + | ND | ND | ND |
| II:1 | 78/F | >500 | X | – | X | Renal adenocarcinoma S/P L nephrectomy | No polyps or CA | + | – | – | – |
| II:2 | 80/F | + | NA | NA | NA | No studies or history | Not done | + | ND | ND | ND |
| II:3 | 96/M | >100 | – | – | – | No CT or US | No polyps or CA | + | ND | ND | ND |
| II:4 | 86/F | + | NA | NA | NA | No studies or history | Not done | + | ND | ND | ND |
| II:5 | 91/M | + | NA | NA | NA | No studies or history | Not done | + | ND | ND | ND |
| III:1 | 45/F | >500 | – | – | – | No CT or US | No polyps or CA | + | – | – | – |
| III:2 | 39/F | >500 | X | X | – | No CT or US | Not done | + | – | – | – |
| III:3 | 53/M | – | – | – | – | No CT or US | No polyps or CA | – | ND | ND | ND |
| III:4 | 53/M | – | – | – | – | No CT or US | Not done | – | ND | ND | ND |
| III:5 | 61/M | – | X | – | – | No CT or US | No polyps or CA | – | ND | ND | ND |
| III:6 | 57/F | >500 | X | – | X | CT of abdomen normal | No polyps or CA | + | – | – | – |
| III:7 | 62/F | >100 | X | – | X | CT of abdomen normal | No polyps or CA | + | – | – | – |
| III:8 | 60/F | – | X | – | – | No CT or US | No polyps or CA | – | ND | ND | ND |
| III:9 | 61/M | >500 | X | X | – | Chromophobe renal CA S/P L nephrectomy | No polyps | + | – | – | – |
| III:10 | 64/M | – | – | – | – | CT of abdomen normal | One polyp, no CA | – | ND | ND | ND |
| III:11 | 53/F | >500 | X | – | – | No CT or US | No polyps or CA | + | ND | ND | ND |
| III:12 | 58/M | >500 | X | – | – | No CT or US | No polyps or CA | + | ND | ND | ND |
| III:13 | 56/F | >500 | X | – | – | No CT or US | No polyps or CA | + | ND | ND | ND |
| III:14 | 72/F | – | X | – | – | No CT or US | No polyps or CA | – | ND | ND | ND |
| III:15 | 48/F | – | – | – | – | No CT or US | No polyps or CA | – | ND | ND | ND |
| III:16 | 80/F | – | X | – | – | No CT or US | No polyps or CA | – | ND | ND | ND |
| III:17 | 71/F | >100 | X | – | X | No CT or US | No polyps or CA | + | ND | ND | ND |
| IV:1 | 17/M | – | – | – | – | No CT or US | Not done | – | ND | ND | ND |
| IV:2 | 23/F | – | – | – | – | No CT or US | Not done | – | ND | ND | ND |
| IV:3 | 19/F | – | – | – | – | No CT or US | Not done | – | ND | ND | ND |
| IV:4 | 41/M | >500 | X | – | – | CT of abdomen normal | No polyps or CA | + | – | – | – |
| IV:5 | 41/F | >500 | – | – | – | No CTor US | Not done | + | – | – | – |
| IV:6 | 35/M | – | – | – | – | No CTor US | Not done | – | ND | ND | ND |
| IV:7 | 35/F | – | X | – | – | No CTor US | Not done | – | ND | ND | ND |
| IV:8 | 33/F | – | – | – | – | No CTor US | Not done | – | ND | ND | ND |
| IV:9 | 52/F | – | – | – | – | No CTor US | One polyp, no CA | – | ND | ND | ND |

*FF, fibrofolliculoma; TD, trichodiscoma; ST, skin tags; OP, oral papules; L, lipomas; BHD, Birt-Hogg-Dube syndrome; NA, data not available; CT, computed tomography; US, ultrasound; L, left; ND, not done; CA, cancer.

### Sequencing

Simultaneous bidirectional sequencing of DNA fragments was performed as previously described.[30] Sequitherm Excel II kit (Epicentre Technologies) and dye labelled primers M13F IRD700 and M13R IRD800 (LI-COR) were used as recommended by the manufacturers. Products were analysed on a 4200S dual dye automated sequencer (LI-COR). Sequences were analysed with Sequencher software (Gene Codes).

### Genotyping

Genetic markers D10S215, D10S1739, D10S541, D10S1427, DS3547, D3S1283, D3S1768, D3S1298, and D3S2432 were typed on the LI-COR 4200S using 25 cm polyacrylamide gels. Sequences of the primers for genetic markers were obtained from GDB and modified so that one primer of each pair was tailed with either M13F or M13R. One µl of DNA sample was amplified in 5 µl reactions containing 0.1 pmol of each of the forward and reverse primers, 0.16 pmol of corresponding fluorescently labelled M13 primer, 0.2 units of Ampli*Taq* (Perkin-Elmer) in PCR buffer containing 2 mmol/l $MgCl_2$ and 200 $\mu$mol/l of each of dNTPs. The amplification procedure consisted of an initial denaturation for two minutes at 94°C followed by 30 cycle of 94°C for 30 seconds, 55°C for 75 seconds, 72°C for 30 seconds, and a final extension for five minutes at 72°C.

### Linkage analysis

We performed linkage analysis using MLINK in the LINKAGE package version 5.1.[31] We used a low penetrance model and assumed that by the age of 40, 10% of carriers would have developed the disease, and after the of age 40, 40% of carriers would have developed the disease.

## RESULTS

The clinical findings of this family are summarised in table 1 and the pedigree is shown in fig 1. Eighteen subjects had multiple white to skin coloured papules ranging from 2-4 mm in diameter (fig 2). Nine subjects had more than 500 lesions and three had more than 100 lesion distributed over their face, neck, and upper trunk. Ten subjects affected with BHD had multiple skin tags. Clinically, five subjects with BHD had multiple lipomas. Two had multiple oral fibromas on their oral mucosa and gingiva. This family has two members with BHD and renal neoplasms. II.1 and III.9 had renal tumours in their left kidney. II.1 presented with haematuria and underwent left nephrectomy at the age of 59. Microscopic examination of renal tissue showed unclassified renal adenocarcinoma. III.9 had an asymptomatic 3.2 cm tumour in the left kidney that was detected by a screening CT scan of the abdomen. He underwent a total left nephrectomy and microscopic examination showed chromophobe renal cell carcinoma (fig 2). We also screened five family members but none had renal tumours. Nineteen family members were examined by colonoscopy. Eleven subjects with BHD underwent colonoscopy to screen them for colonic polyps and tumours, with negative results. Only two of eight family members who were not affected with BHD had solitary benign polyps.

### Molecular findings

The results of the mutation analysis are summarised in table 1. No germline *PTEN*, *PTCH*, or *CTNNB1* mutations were found

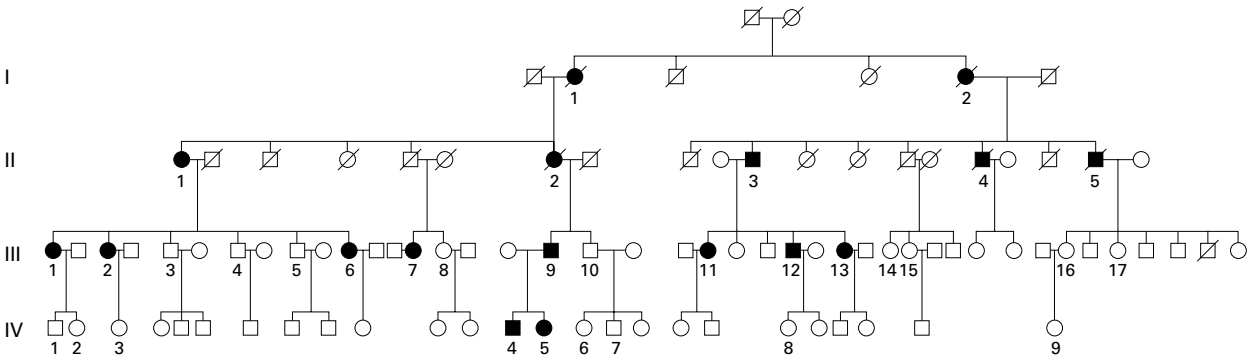


**Figure 1** Pedigree of family affected with Birt-Hogg-Dube (BHD) syndrome. Filled symbols = affected with BHD.

in eight subjects with BHD whose DNA was analysed. In linkage analysis for *CTNNB1* and *PTEN*, all markers had a lod score of –2.0 to –3.0. Therefore, there was no linkage to 3p21 or 10q21, the loci for *CTNNB1* and *PTEN*, respectively. These data suggest that *PTEN*, *PCTH*, and *CTNNB1* are excluded as candidate genes for *BHD*.

## DISCUSSION

In this study, we report an extended family with BHD and renal cancer in which various molecular methods were used to evaluate *PTCH, PTEN*, and *CTNNB1* as candidate genes for BHD. These genes were studied because mutations in each are associated with a disorder that has clinical overlapping features with BHD and these disorders carry an increased risk for internal malignancy. Sequencing analysis indicated no mutations in *CTNNB1, PTCH*, or *PTEN* genes and there was no linkage to 10q23, the locus for *PTEN*, or 3p21, the locus for *CTNNB1*.

Of the candidate genes we tested, the gene for Cowden syndrome was considered most likely, as Cowden syndrome shares the most clinical features with BHD. Cowden syndrome and BHD usually consist of multiple hamartomas of the skin and mucous membranes. Patients with either BHD or Cowden syndrome present with hamartomas of the hair follicles. Multiple facial tricholemmomas, which are tumours of the follicular infundibulum, are distinctive lesions associated with Cowden syndrome.[20] In contrast, fibrofolliculomas, hamartomas of the outer root sheath of the hair follicles, are characteristic of BHD. Multiple fibromas of the oral mucosa give a cobblestone-like appearance of the gingiva, palate, and buccal mucosa and are present in both BHD and Cowden syndrome. In this study, multiple lipomas were present in four subjects with BHD. Multiple lipomas have also been reported to occur in patients with BHD and Cowden syndrome.[20] However, palmoplantar keratoses and storiform fibromas, which are commonly present in Cowden syndrome,[20] were not identified in this BHD family.

*P*hosphatase and *ten*sin homologue deleted on chromosome 10 (*PTEN*), the gene for Cowden syndrome, has been mapped to 10q23.3.[20] We performed linkage with loci at 10q23.3 and mutational analysis of the *PTEN* gene in our family. We found no linkage and sequencing of the individual exons of *PTEN* did not show mutations. In contrast to BHD, only a few patients with Cowden syndrome and *PTEN* mutations have been reported to have renal cancer. March *et al*[21] found renal cell

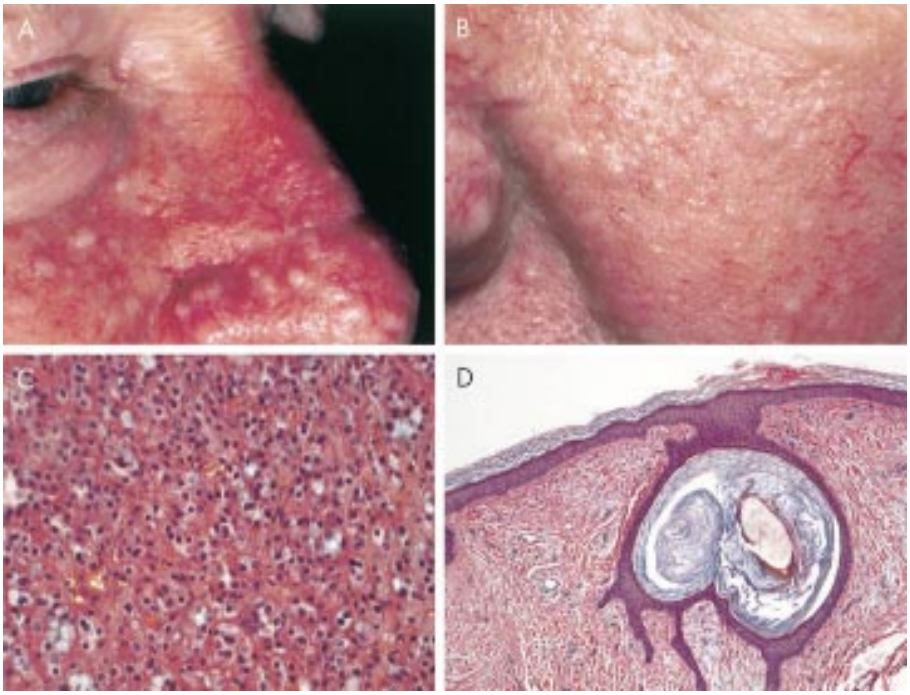


**Figure 2** Clinical and histological features of fibrofolliculomas and a renal tumour of III.9. (A) Multiple fibrofolliculomas on the nose and paranasal areas. (B) Fibrofolliculomas on the cheek. (C) Histopathology of a renal tumour after total left nephrectomy. Microscopic examination showed chromophobe renal cell carcinoma. (D) Histopathology of a fibrofolliculoma shown in (A). Multiple anastomosing strands of 2-4 epithelial cells extend from the central follicle.

carcinomas (RCC) in two men with Cowden syndrome who had germline mutations in *PTEN*. One had clear renal cell carcinoma and the other had papillary renal cell carcinoma (C Eng, personal communication, 1998). A case of Cowden syndrome associated with RCC and Merkel cell carcinoma has also been reported.[32] Somatic *PTEN* mutations have been found at a low frequency in primary RCCs and RCC cell lines. Steck *et al*[33] reported a somatic *PTEN* mutation in one of six primary RCCs. Alimov *et al*[34] found somatic *PTEN* inactivation in three of 54 primary RCCs and one of nine RCCs cell lines. Recently, Kondo *et al*[35] also found *PTEN* mutations in five of 68 primary RCCs and one of 17 cell lines. It is likely that *PTEN* may play a role in tumorigenesis in a subgroup of RCC. In this study, sequencing analysis did not show *PTEN* germline mutations in two patients with BHD and RCC. In this study one subject with BHD had chromophobe RCC. The histopathology was similar to the one reported by Roth *et al*[5] of a 61 year old man with BHD and bilateral renal cell carcinoma. Recently, Toro *et al*[2] excluded *VHL* and the tyrosine kinase domain of the *MET* proto-oncogene as candidate genes in two BHD families with renal oncocytomas and one BHD family with a variant of papillary renal cell carcinoma. This study and previous reports suggest that BHD is associated with a spectrum of renal tumours ranging from chromophobe to oncocytoma to clear cell renal carcinoma. The spectrum of renal tumours associated with BHD suggests that the BHD gene may play an important role in renal embryogenesis.

We did not find colonic polyps or carcinoma in any of the subjects with BHD who were screened by colonoscopy. In addition, none had a history of colon cancer or polyps. This is in contrast to previous reports.[3 4] However, multiple hamartomatous polyps of the gastrointestinal tract are seen as a component of Cowden syndrome.[21] We found two unaffected subjects with a solitary polyp. Colonic polyps are common findings in the normal population. Therefore, it would not be unusual to find that some unaffected subjects had colonic polyps. Our findings and previous reports suggest that BHD is not associated with medullary thyroid carcinoma. In contrast to BHD, hamartomas and non-medullary cancer of the thyroid are commonly seen in Cowden syndrome.[20]

BCNS and BHD share the clinical presentation of multiple facial papules and an increased risk of internal malignancy. Multiple basal cell carcinomas, odontogenic keratocysts, and palmoplantar pits constitute the primary features of Gorlin syndrome.[22] Typically, multiple basal cell carcinomas develop at a young age in BCNS. However, two of our BHD patients developed multiple facial BCCs during late adulthood. None of our patients with BHD had palmoplantar pits or odontogenic keratocyst and none had a history of medulloblastoma, ovarian fibromas, or other features associated with Gorlin syndrome.[22] Superficial milia and deeper cysts may be present in both BCNS and BHD. Gorlin syndrome is caused by mutations in *PTCH*,[23] which is localised to 9q22.3. *PTCH* is the human homologue of *Drosophila patched*. Bidirectional dideoxy fingerprinting analysis of *PTCH* in 11 subjects with BHD excluded *PTCH* as a candidate gene for BHD.

*CTNNB1* appeared to be a good candidate gene for BHD for various reasons. Mutations in *CTNNB1*, the gene for β-catenin, have been reported in most pilomatricomas.[24] Fibrofolliculomas and pilomatricomas are benign tumours derived from the hair follicle. While pilomatricomas are tumours of the hair matrix,[36] fibrofolliculomas are hamartomas of the outer root sheath of the hair follicle. The WNT pathway plays an important role in development, carcinogenesis, and hair follicle formation.[37] β-catenin enables downstream transcription activation by LEF/TCF in the WNT pathway.[38 39] Most colon cancers have mutations in adenomatous polyposis coli (*APC*).[40] *APC* codes for a protein required for ubiquitin mediated degradation of β-catenin. Recent studies suggest that the WNT gene family is likely to be an important mediator of the transformation of the mesenchyme to epithelium in kidney embryogenesis.[26 27] Stark *at al*[41] reported that the expression of Wnt-4 correlates with, and is required for, kidney tubulogenesis and mice lacking Wnt-4 activity fail to form pretubular cell aggregates. Thus, Wnt-4 appears to act as an autoinducer of the mesenchyme to epithelial transition that underlies nephron development. Similarly, Nguyen *et al*[27] showed that Wnt-4 and -11 are likely to be important mediators of the transformation of mesenchyme to epithelium in the kidney. Obstruction induced during metanephrogenesis disrupts the normal pattern of Wnt-4, -7b, and -11 expression and interferes with the normal transformation process in developing kidneys, by maintaining the mesenchymal component and inducing the transformation of epithelium to mesenchyme. Furthermore, several groups have reported that a small percentage of renal cancers have mutations in *CTNNB1*. Kim *et al*[26] reported aberrant expression of β-catenin protein and a mutation of exon 3 of *CTNNB1* in renal cancer. They reported that cytoplasmic accumulation of β-catenin was observed in 22.7% (5/22) cases of clear RCC, but not in papillary or chromophobe renal carcinomas. They identified a missense mutation in codon 61 of *CTNNB1* in one case of RCC. However, recent work by Bilim *et al*[42] failed to show mutations in the third exon of the *CTNNB1* in RCC. These studies indicate that mutations of *CTNNB1* are relatively rare events in RCC.

- Renal cancer often occurs as part of Birt-Hogg-Dube syndrome. We present a family with 18 members affected with Birt-Hogg-Dube syndrome. This family had two subjects with BHD and renal cancer. Eleven subjects with BHD underwent colonoscopy to screen them for colonic polyps and tumours, with negative results.
- No germline *PTEN*, *PTCH*, or *CTNNB1* mutations were found in eight subjects affected with BHD whose DNA was analysed. There was no linkage to 3p21 or 10q21, the loci for *CTNNB1* and *PTEN*, respectively. These data suggest that *PTEN*, *PCTH*, and *CTNNB1* are excluded as candidate genes for BHD.

Canine genetics may shed some light on the chromosomal location of a new gene responsible for renal cancer and skin hamartomas similar to BHD. Renal cancer is rare in domestic animals and only a few cases have been reported in dogs. In 1985, Lium and Moe[43] reported German Shepherd dogs in which multifocal renal cystadenocarcinoma and nodular dermatofibrosis (RCND) were inherited in an autosomal dominant fashion. Histologically, nodular dermatofibrosis consists of dense deposition of collagen fibres very similar to the stromal changes in trichodiscoma and collagenoma. BHD and this group of German Shepherd dogs share many features in common: autosomal dominant inheritance, renal cysts and tumours, and a dense collagen deposition in the skin. Therefore, German Shepherd dogs with RCND may be a good animal model for BHD. Recently, Jonasdottir *et al*[44] used genetic linkage analysis of a pedigree of German Shepherd dogs with RCND to localise the disease to a small region of canine chromosome 5. The closest marker, CO2608, is linked to RCND with a lod score of 16.7. CO2608 and the adjacent linked markers map the region of the genome homologous to human chromosomes 1p and 17p. This makes chromosome 1p and 17p the most likely candidate regions for BHD. Phenotypic similarities between RCND and some human disorders have suggested several other candidate genes. Tuberous sclerosis complex (TSC) has an autosomal dominant mode of inheritance and is caused by mutations in the *TSC1* and *TSC2* genes.[45] Both TSC and RCND share connective tissue hamartomas. Although RCND bears some similarities to TSC, the renal carcinomas associated with TSC are typically hamartomas or angiomyolipomas, whereas those associated with RCND are cystadenocarcinomas. Interestingly, the clinical syndrome presented by the Eker rat also shows

some similarity to RCND, with kidney tumours and reproductive tract leiomyomas being common in both diseases.[46 47] Both TSC genes have been excluded as candidate genes for RCND by canine linkage studies. The mapping of RCND shows the potential of the dog in mapping genes for human genetic disorders. Identification of gene RCND could make a unique contribution to our understanding of kidney and skin biology as well as cancer susceptibility.

In conclusion, we excluded *PTEN*, *CTNNB1*, and *PTCH* as candidate genes for BHD. Canine linkage studies for RCND suggest that human chromosomal regions 1p and 17p are the most likely candidate regions for the BHD gene.

**Authors' affiliations**

**J R Toro,** National Cancer Institute, National Institutes of Health (NIH), Bethesda, Maryland, USA
**J R Toro, Y O Shevchenko, J G Compton, S J Bale,** Genetic Studies Section, Laboratory of Skin Biology, National Institute of Arthritis and Musculoskeletal and Skin Diseases, NIH, Bethesda, Maryland, USA

Correspondence to: Dr J R Toro, National Institutes of Health, Building 10, Room 12N-238, 10 Center Drive, MSC 1908, Bethesda, Maryland 20892-1908, USA; torojo@exchange.nih.gov

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