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

In 1977, Birt, Hogg, and Dube described a kindred in which 15 of 70 members over three generations exhibited multiple, small, grey colour, dome shaped papules distributed over the face, neck, and upper trunk inherited in an autosomal dominant pattern. 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.

The cutaneous manifestations of BHD, which typically appear during the third or fourth decade of life, have been associated with renal carcinoma, spontaneous pneumothorax, and colonic polyps. Toro et al 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 hamangioblastomas of the brain, spine, and eye owing to mutations in the von Hippel-Lindau (VHL) disease tumour suppressor gene; (2) clear cell renal carcinoma associated with constitutional, balanced translocations involving the short arm of chromosome 3; (3) papillary renal cell carcinoma associated with germ-line mutations in the tyrosine kinase domain of the MET proto-oncogene; and (4) renal oncocytoma.

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, 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.

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. 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. The clinical presentation of multiple facial follicular 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. Sequence specific PCR primers were designed based on published sequences, with Tms adjusted to approximately 60°C. The sequences M13F (CAGACGTTGTAAAACGAC) and M13R (GGA-TAACATTTCCACAGGG) 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 RedTaq 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.

Abbreviations: BHD, Birt-Hogg-Dube syndrome; BCNS, basal cell naevoid syndrome; RCC, renal cell carcinoma; RND, renal cystadenocarcinoma and nodular dermatofibrosis; TSC, tuberous sclerosis complex.
Sequencing
Simultaneous bidirectional sequencing of DNA fragments was performed as previously described. 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 LI-COR 4200S using 25 cm polyacrylamide gels. Sequences of the primers for genetic markers were obtained on the LI-COR 4200S.

Genotyping
Genetic markers D10S215, D10S1739, D10S541, D10S1427, D3S3547, D3S1283, D3S1768, D3S1298, and D3S2432 were typed on the LI-COR 4200S using 25 cm polyacrylamide gels. Genetic markers D10S215, D10S1739, D10S541, D10S1427, D3S3547, D3S1283, D3S1768, D3S1298, and D3S2432 were typed on the LI-COR 4200S using 25 cm polyacrylamide gels.

Linkage analysis
We performed linkage analysis using MLINK in the LINKAGE package version 5.1. 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 age of 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).

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

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*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.
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, PCH, 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 trichoellemomas, which are tumours of the follicular infundibulum, are distinctive lesions associated with Cowden syndrome. 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. However, palmoplantar keratoses and storiform fibromas, which are commonly present in Cowden syndrome, were not identified in this BHD family.

Phosphatase and tensin homologue deleted on chromosome 10 (PTEN), the gene for Cowden syndrome, has been mapped to 10q23.3. 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 found renal cell

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

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 cancer 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. Somatic PTEN mutations have been found at a low frequency in primary RCCs and RCC cell lines. Steck et al. reported a somatic PTEN mutation in one of six primary RCCs. Alikun et al. found somatic PTEN inactivation in three of 54 primary RCCs and one of nine RCCs cell lines. Recently, Kondo et al. 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 gene deletions or 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. of a 61 year old man with BHD and bilateral renal cell carcinoma. Recently, Toro et al. 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. However, multiple hamartomatous polyps of the gastrointestinal tract are seen as a component of Cowden syndrome. 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.

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 palmpoplantar pits constitute the primary features of Gorlin syndrome. Typcially, 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 palmpoplantar pits or odontogenic keratocyst and none had a history of medulloblastoma, ovarian fibromas, or other features associated with Gorlin syndrome. Superficial milia and deeper cysts may be present in both BCNS and BHD. Gorlin syndrome is caused by mutations in PTCH, 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. CO2608 and the adjacent linked markers map the location of a new gene responsible for renal cancer and skin hamartomas (RCND) to a small region of canine chromosome 5. CO2608 and the adjacent linked markers map the location of a new gene responsible for renal cancer and skin hamartomas (RCND) to a small region of canine chromosome 5. This makes chromosome 1p and 17p the most likely locations for the BHD gene. In one case of RCC, Kim et al. 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. 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.

Canine genetics may shed some light on the chromosomal location of a new gene responsible for renal cancer and skin hamartomas in humans. Canine genetics may shed some light on the chromosomal location of a new gene responsible for renal cancer and skin hamartomas in humans. The canine equivalent of the BHD gene may be on the canine chromosome 15 which is the homologue of human chromosome 17. Canine genetics may shed some light on the chromosomal location of a new gene responsible for renal cancer and skin hamartomas in humans. The canine equivalent of the BHD gene may be on the canine chromosome 15 which is the homologue of human chromosome 17. Groups 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 may be a good animal model for BHD. Recently, Jonasdotir et al. 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 1p. This makes chromosome 1p and 1p 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. 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

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, PCH, 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.
some similarity to RCND, with kidney tumours and reproductive tract leiomyomas being common in both diseases.” Both the genes harbouring the disease-causing mutations are called PTEN 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 PTTCH 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.

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