Novel Mutations in the *BHD* Gene and Absence of Loss of Heterozygosity in Fibrofolliculomas of Birt-Hogg-Dubé Patients

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Birt-Hogg-Dubé (BHD) syndrome is an autosomal-dominantly inherited cancer syndrome characterized by fibrofolliculomas, lung cysts leading to pneumothorax, and chromophobic/oncocytic renal cell carcinoma. The disease is caused by heterozygous mutations in the *BHD* gene encoding folliculin and all mutations reported putatively lead to protein truncation. Although the function of folliculin is unknown, it is thought to be a tumor suppressor, with loss of heterozygosity (LOH) initiating tumor formation. Here, we report on four novel *BHD* gene mutations, including two splice-site mutations, in patients presenting with skin lesions only. We further show that LOH cannot be detected in fibrofolliculomas from three patients, suggesting that for the manifestation of cutaneous tumors in BHD syndrome haplo-insufficiency of folliculin is sufficient to initiate uncontrolled growth. Renal microscopic oncocytosis in BHD is considered as a precursor to malignant kidney tumors and may likewise be the result of haplo-insufficiency, with somatic second-hit mutations or LOH giving rise to malignancy later in life.

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INTRODUCTION

Birt-Hogg-Dubé (BHD) syndrome (OMIM 135150) is a rare tumor syndrome that is inherited in an autosomal dominant manner. The clinical hallmarks of the disease are fibrofolliculomas, benign hair follicle tumors with a typical histology, consisting of anastomosing epithelial strands surrounding a fibrovascular stroma (Scalvenzi *et al.*, 1998). Besides the cutaneous signs, pneumothorax is a common feature of the disorder, with the highest incidence before the age of 40 years (Zbar *et al.*, 2002). One study reported on a BHD family that apparently presented with pneumothorax exclusively (Painter *et al.*, 2005). Additionally, the occurrence of chromophobic/oncocytic hybrid and pure chromophobic kidney tumors has been reported, mostly affecting both kidneys and predominantly manifesting after the age of 40 years (Zbar *et al.*, 2002).

The disease is caused by mutations in the *BHD* gene that encodes folliculin, a cytoplasmic protein with a predicted size of 64 kDa. Alternative splicing of the *BHD* gene results in two isoforms of folliculin, a full-length form and one that uses an initiation codon in intron 6 (Nickerson *et al.*, 2002). The function of folliculin and its alternate splice variant is presently unknown. All germline mutations in patients suffering from BHD syndrome described so far are splice-site mutations or insertions/deletions that likely result in protein truncation and haplo-insufficiency. In this study, we identified five mutations in BHD syndrome patients of which four are novel. These include two deletions and one insertion that lead to frameshifts and two splice-site mutations. RNA studies revealed that one splice-site mutation results in the absence of identifiable aberrant reverse transcriptase-PCR (RT-PCR) products, whereas the other one leads to exon skipping.

Previously, the occurrence of loss of heterozygosity (LOH) at the BHD locus has been studied in sporadic kidney tumors. From these data, it appears that LOH of the *BHD* gene can be found in a minority of such sporadic renal malignancies (Khoo *et al.*, 2003). In BHD patients though, second-hit somatic mutations appear to be more frequent (Vocke *et al.*, 2005). Despite these data obtained in renal tissue, it has not yet been studied if folliculin also behaves as a typical tumor suppressor in the skin. To determine whether LOH or second-hit somatic mutations play a role in the pathogenesis of the cutaneous tumors arising in BHD syndrome, we examined fibrofolliculomas from three different patients. Our data suggest that LOH or second-hit somatic mutations are most likely not involved in the pathogenesis of these skin tumors.

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Abbreviations: BHD, Birt-Hogg-Dubé; LOH, loss of heterozygosity; RT-PCR, reverse transcriptase-PCR

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RESULTS

Mutation analysis

In all patients studied we detected mutations in the *BHD* gene, consisting of one insertion, two deletions, and two splice-site mutations (one at the donor, the other one at the acceptor splice site), designated 1277insC, 420delC, 1408_1418delGGGAGCCCTGT, IVS10-2A>G, and 1300G>C, respectively (Table 1). These sequence deviations were absent in 96 healthy control individuals as confirmed by restriction enzyme digestion. To analyze the consequences of the two latter mutations, we performed RNA analyses.

RT-PCR analyses

In patients MH63 and MC60 carrying the mutations 1300G>C and IVS10-2A>G, respectively, we first performed RT-PCR in order to study the specific splicing pattern and the consequences arising from these sequence deviations on the cDNA level.

Mutation 1300G>C theoretically results in the substitution of a glutamine residue by a glutamic acid residue with the putative designation E434Q. Because it affects the last coding nucleotide of exon 11, we reasoned that this mutation might result in aberrant mRNA splicing. This hypothesis was subsequently confirmed by demonstrating skipping of exon 11 in patient MH63 as a consequence of this mutation (Figure 1). In patient MC60, we did not detect any aberrant PCR products (data not shown).

LOH studies

In fibrofolliculomas obtained from patients MH63, MC60, and MMC59, we sought for LOH or second-hit mutations on the DNA level. Comparative sequence analyses for all coding exons of *BHD* performed on DNA extracted from a skin tumor biopsy *versus* genomic DNA isolated from peripheral blood lymphocytes did not reveal any sequence deviations indicative of LOH or second-hit mutations. The mutations found in leukocyte DNA were also present in the heterozygous state in DNA derived from the fibrofolliculomas. To further rule out the presence of LOH, we performed laser capture microdissection on a tumor sample from individual TQ27 (Figure 2a). Multiple tumor fields were dissected and in the DNA isolated from all of those, we found the 11 bp deletion in a heterozygous state (Figure 2b). To determine if LOH occurs through promoter hypermethylation, DNA from lymphocytes as well as from fibrofolliculomas was tested with a methylation-sensitive PCR and subsequent sequencing. We could not detect any differences between sequence traces from the three fibrofolliculoma biopsies and from lymphocytes.

DISCUSSION

Here, we studied five patients with BHD syndrome and detected five distinct sequence deviations, including one insertion, two deletions, and two splice-site mutations. Of these, four are novel. Mutation 1277insC has been described previously as 1733insC (Nickerson *et al.*, 2002). Our numbering differs because we take the first nucleotide of the initiation codon as a starting point (exon 4), instead of the first non-coding exon of the *BHD* gene. We also sought for evidence of LOH in fibrofolliculomas from three of these patients.

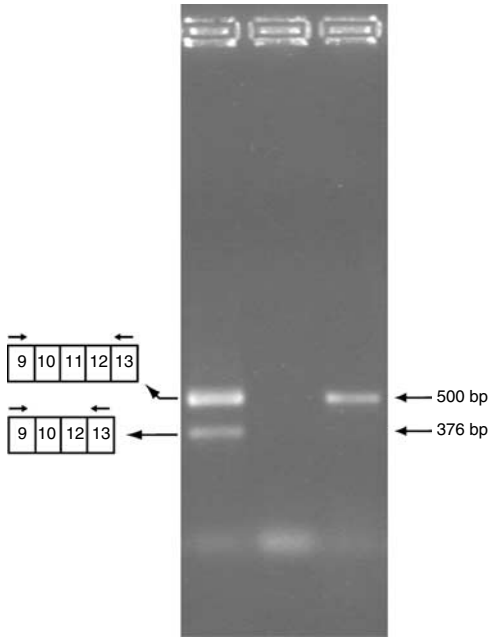

Figure 1. RT-PCR results from a fibrofolliculoma biopsy of patient MH63 with primers BHD9cF and BHD13cR2. Lane 1, mutant; lane 2, −RT control; lane 3, wild-type. The −RT control is negative, ruling out contamination with genomic DNA. Sizes of the PCR products are on the right and illustrations representing the products on the left.

Table 1. Mutations identified in the *BHD* gene of BHD patients

Patient	Exon/intron	Nucleotide change	Codon location	Mutation type
TQ27	Exon 12	1408_1418delGGGAGCCCTGT	G470	FS
MH63	Exon 11	1300G>C	E434	SS
MC60	Intron 10	IVS10-2A>G	—	SS
MMC59	Intron 10	IVS10-2A>G	—	SS
MB61	Exon 6	420delC	P140	FS
KD63	Exon 11	1277insC	H429	FS

BHD, Birt-Hogg-Dubé; FS, frameshift; SS, splice site.

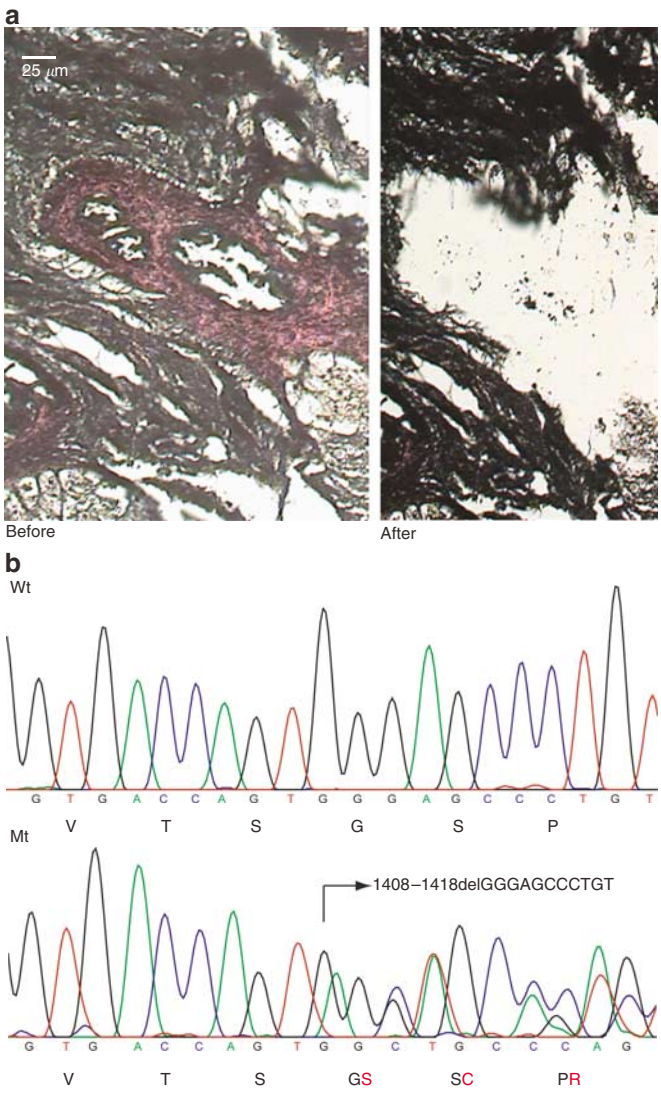

Figure 2. Micrographs of the laser capture microdissection procedure. The fibrofolliculoma is colored magenta. The panel labeled ''after'' shows the situation after dissection of one tumor field, with the tumor tissue removed. Bar = 1 mm = 2.5 μm. (**a**) Sequence chromatogram of exon 11, obtained from the microdissected tumor cells of patient TQ27 (PCR and sequencing with primers BHD12F and BHD13cR). The 11 bp deletion is clearly present in the heterozygous state.

BHD syndrome shows considerable clinical and genetic heterogeneity, which is reflected by previous data as well as our own observations in this study. The symptoms encountered in our patients and their family histories confirm other reports in which BHD syndrome is shown to be clinically heterogeneous, with kidney tumors being relatively rare (Schmidt *et al.*, 2005). Although one study suggests that pneumothorax can be the only clinical sign (Graham *et al.*, 2005; Painter *et al.*, 2005), we consider this rather unlikely, based on our own experience with several patients and their families, because all affected persons examined by us developed folliculomas after the age of 35 years. These skin lesions might easily escape detection because they are rather subtle. With this in mind, it probably would be of interest to re-examine the family reported by Painter *et al.* (2005) (Graham *et al.*, 2005).

To date, several mutations in the *BHD* gene have been reported, including insertions, deletions, and splice-site mutations. About 40–50% are insertions/deletions in a hypermutable C(8)-tract in exon 11 (Lamberti *et al.*, 2005). The two deletion mutations, 420delC and 1408_1418delGGGAGCCCTGT (Table 1), cause frameshifts and premature termination codons and might lead to haplo-insufficiency as a result of protein truncation or nonsense-mediated mRNA decay.

The mutation in MH63 is interesting, because it affects the last nucleotide of exon 11, changing a G to a C, thus presumably resulting in a missense mutation, E434Q. However, the splicing pattern of our subsequent RT-PCR analysis showed that the mutation affected mRNA splicing, causing the skipping of exon 11. The second splice-site mutation detected in patient MC60 might result in partial inclusion of intron 11 with frameshift (NetGene2, http://www.softberry.com) and subsequent nonsense-mediated mRNA decay initiated by stop codons in the mRNA, as reported previously (Zeniou *et al.*, 2004). In support of this prediction, we were not able to demonstrate any mutant mRNA splice products upon RT-PCR.

All mutations reported in BHD syndrome, including the ones described here, are predicted to result in haplo-insufficiency. Interestingly, neither in humans nor in the Nihon rat model of BHD (Okimoto *et al.*, 2004), missense mutations have been described yet. By contrast, however, in a canine model of the disease, a missense mutation in the *BHD* gene has been reported (Lingaas *et al.*, 2003). The mutated histidine (H255) in the canine folliculin protein is highly conserved among various mammalian species, including mouse, rat, cow, dog, and human, suggesting that it is crucial to protein function. Mutations that cause BHD syndrome-like symptoms in the dog might therefore be expected to cause loss-of-function of the mutated allele. Because the canine phenotype differs from the human one in certain aspects, it would also be conceivable that it is not caused by haplo-insufficiency. Elucidation of the function of folliculin will help to resolve this matter.

It has been suggested that folliculin behaves as a typical tumor suppressor protein, with loss-of-function of the remaining, healthy allele resulting in tumorigenesis. However, the results of our RT-PCR analyses of tumor biopsies in two patients showed that at least in one patient (MH63) biallelic gene expression was always present. These data indicate that LOH or promoter hypermethylation was absent in the bulk of the skin lesions. In the other patient (MC60), we could only demonstrate the presence of a wild-type allele. We can rule out the possibility of promoter hypermethylation in the remaining healthy allele within the tumor based on the results of the bisulfite PCR, also in the other two patients thus examined. Analysis of DNA obtained from tumor biopsies from three patients further suggests that somatic second-hit mutations are not present. Finally, laser capture microdissection of tumor tissue convincingly demonstrated heterozygosity for the germline mutation, demonstrating that low

numbers of healthy cells in the analysis of total biopsy material are not obscuring the results. Of note, LOH was only detected in a minority of BHD syndrome-associated renal malignancies (Khoo *et al.*, 2003) to date, whereas comparable studies in the characteristic skin tumors associated with this disease have, to the best of our knowledge, not been carried out yet. In most BHD syndrome-associated malignant kidney tumors, the healthy allele is lost by somatic mutations (Vocke *et al.*, 2005) that were not detectable in fibrofolliculomas of our patients. Our results obtained in fibrofolliculomas from BHD patients suggest that tumorigenesis in this syndrome does not necessarily reflect the consequence of a complete loss of folliculin function. Rather, haplo-insufficiency may be sufficient to cause uncontrolled growth and subsequent carcinogenesis in susceptible tissues. If this is also the case for the renal microscopic oncocytic proliferations in BHD syndrome (Pavlovich *et al.*, 2002) remains to be determined. These lesions are thought to be the precursors to malignant kidney tumors. From our findings, we would expect microscopic oncocytosis to be heterozygous for the *BHD* germline mutation.

MATERIALS AND METHODS

Case reports

All patients initially presented for diagnosis and treatment of flesh-colored papules on the face that occurred from age 30–35 years onward (Figure 3, patient MC60). The specific details of each patient, including the family history, are listed in Table 2. Of note, one patient had been misdiagnosed with acne vulgaris first and, therefore, treated with isotretinoin, leading to a reportedly significant regression of the skin lesions. None of the patients had suffered from pneumothorax or renal carcinomas at the moment of presentation, although patient KD63 had a history of spontaneous pneumothorax. Thoracic X-ray examination was normal in all patients and screening for renal malignancies by magnetic resonance imaging did not reveal pathological findings in any of the patients. Although there are no established guidelines for follow-up in BHD syndrome and kidney tumors typically develop from the fifth decade of life onward (Pavlovich *et al.*, 2005), we did offer our patients yearly screening by abdominal ultrasound examination.

In every patient, a skin biopsy for histological examination was taken. A representative image (from patient TQ27) is depicted in Figure 4. All biopsies revealed anastomosing epithelial strands that are characteristic of fibrofolliculomas, with the majority of the biopsy volume typically occupied by the tumor. To study the splicing pattern of *BHD* mRNA and for the detection of possible LOH, we isolated RNA and DNA from the tumors of three patients, MH63, MC60, and MMC59.

We obtained informed consent from the patients for all experimental procedures in accordance with institutional requirements and the Declaration of Helsinki Principles.

PCR and mutation detection

DNA was isolated from peripheral blood samples using a salt precipitation technique described elsewhere (Miller *et al.*, 1988). All coding regions and the adjacent splice sites of the *BHD* gene were amplified by PCR (primer sequences are listed in Table 3). PCR

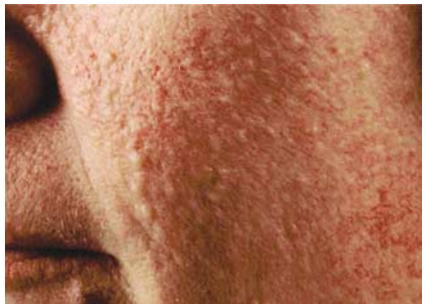

Figure 3. Patient MC60. Typical distribution of numerous flesh-colored papules in the face.

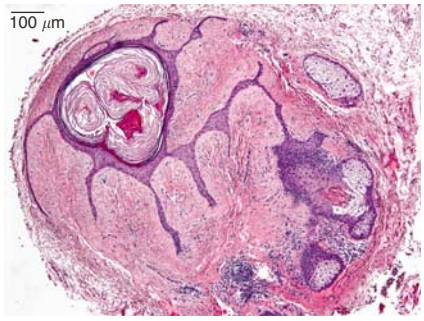

Figure 4. The typical histology of a fibrofolliculoma, with its anastomosing strands of epithelial cells. Bar = 1 mm = 100 μm. The sample was obtained from patient MC60.

Table 2. Patient characteristics

Patient	Age at presentation (years)	Presenting symptom	Family history	Pneumothorax	Kidney MRI	Thorax X-ray
TQ27	77	Fibrofolliculoma	Two affected sons, multiple other affected family members	No	Normal	Normal
MH63	42	Fibrofolliculoma	Negative	No	Normal	Normal
MC60	45	Fibrofolliculoma	Sister (MMC59) affected	No	Normal	Normal
MB61	44	Fibrofolliculoma	Negative	No	Normal	Normal
KD63	42	Fibrofolliculoma	Negative	Yes	Normal	Normal

MRI, magnetic resonance imaging.

Table 3. Primer sequences

Name	Sequence (5′–3′)	PCR product size (bp)
BHD4F	AGGTGCTCCCTGTGCTCCAG	440
BHD4R	CCGTCCACTGCTCTCAGGTC	—
BHD5F	CCGAGCTCAGATTTGCATAAACC	326
BHD5R	CCTGCCTCCCTGTGCAATG	—
BHD6F	TGATTTGTGCCAGCTGACTCTG	400
BHD6R	CCAGGCCTCAACCTCAGCAC	—
BHD7F	CCTGGAGTTGGCTGTGAACG	327
BHD7R	TCCCAAATCCATGGACAAGC	—
BHD8F	GTTGTGCCCTGCTGGTGTTC	315
BHD8R	TTCCCTCCCTCAGCGATTCC	—
BHD9F	GGCCGCAGCCAGGAATCTAC	394
BHD9R	GTGGAGGGTCCAGAGGCAAG	—
BHD10F	CACCCGCCTCCCTGAGAAG	391
BHD10R	CCAGTGGAGACCGTGTGGTG	—
BHD11F	GGTTCCACTTTGGGCCTGAG	289
BHD11R	AGGAGGCGTGTGGGGTTTG	—
BHD12F	CTAGCGCAGGGGAGGTGAGG	478
BHD13R	ACGGCCCAGCTCCTCTTTTG	—
BHD14F	CCGTGTCACCCCTGGTTGG	400
BHD14R	TGCTGGGACACAGCTCCTTC	—
BHD10cF	TCTGGAAAAGCAGAGACGTGGAC	355 (cDNA)
BHD13cR	TGGTTGGTCAGAGCCGCTTC	—
BHD9cF	CCCCGGAAGCTGCCAGTC	500 (cDNA)
BHD13cR2	CAGACGAGGCACTGGTCCAC	—

reactions (25 μl) contained 1 × buffer (Qiagen Benelux BV, Venlo, The Netherlands), 0.2 mM dNTPs, 100 ng of each primer, 3% DMSO, and 0.75 U *Taq* DNA polymerase (Qiagen). PCR conditions were; 30 seconds 94°C, 30 seconds 62°C, 60 seconds 72°C for 35 cycles, initiated by 90 seconds at 94°C, and terminated by 7 minutes at 72°C. PCR products were purified with a MultiScreen-PCR filter plate (Millipore, Billerica, MA) and subsequently directly sequenced (BigDye deoxy terminator V3.1 cycle sequencing kit, Applied Biosystems, Foster City, CA) with the aforementioned PCR primers, according to the specifications of the manufacturer. Sequence analysis was performed with the software tools Phred, Phrap, and Consed (Ewing and Green, 1998; Ewing *et al.*, 1998; Gordon *et al.*, 1998).

The four novel mutations detected herein were excluded in 96 healthy controls by enzymatic digestion analysis. Mutation 420delC was confirmed with restriction endonuclease *Eco*O109I; 1408_1418delGGGAGCCCTGT with *Alw*NI; IVS10-2A>G with *Ava*II; and 1300G>C with *Ale*I, according to the manufacturer's instructions (all enzymes by New England Biolabs, Ipswich, MA).

RT-PCR and LOH studies

Skin punch biopsies (3 mm) of facial lesions were taken, after obtaining informed consent from the patients. The tissue samples were snap frozen in liquid nitrogen. RNA was extracted using the RNEasy mini kit from Qiagen, according to the manufacturer's recommendation. The tissue was homogenized, without prior thawing, with a high-speed stationary homogenizer. First-strand synthesis was performed using the Superscript first-strand synthesis system (Invitrogen, Groningen, The Netherlands) according to the manufacturer's instructions. PCR was then performed following the protocol outlined above. RT-PCR products were analyzed on a 1.5% agarose gel. Individual PCR products were isolated and directly sequenced.

For LOH analysis, DNA was isolated from snap-frozen biopsies using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN) according to the manufacturer's recommendations. Genomic DNA of the biopsy material was subjected to PCR and direct sequencing as described above.

Tissue processing for laser capture microdissection: Four micrometers tissue sections were incubated overnight at 56°C, deparaffinated with xylene, washed with 100% ethanol, stained with hematoxylin, washed with water, dehydrated with increasing percentage of ethanol and subsequently thoroughly dried.

Laser capture microdissection was performed on an Arcturus PixCell II (Arcturus Engineering Inc., Mountain View, CA) microscope using Arc200 version 2.0.0 (Arcturus Engineering Inc.) imaging software. Approximately 100 ejected cells were captured on plastic cups and collected in tissue lysis buffer with proteinase K. Subsequently, DNA was extracted according to the manufacturer's protocol (DNeasy Tissue Kit, Qiagen). The DNA was subjected to PCR using the conditions and primers outlined above. Multiple tumor fields were dissected to allow for possible regional differences in LOH.

The presence of promoter hypermethylation was assessed by methylation-specific PCR as described previously (Herman *et al.*, 1996). We used the EZ DNA Methylation kit by Zymo Research (Orange, CA) according to the manufacturer's recommendations with primers and conditions as described by Khoo *et al.* (2003). Briefly, bisulfite converts unmethylated cytosines to uracil, whereas methylated cytosine remains unchanged. The modified DNA is then amplified by PCR. The methylated allele can be recognized by comparing its restriction pattern or sequence with the unmethylated allele.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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