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Birt-Hogg-Dubé (BHD) syndrome: report of two novel germline mutations in the folliculin (FLCN) gene

Molecular analysis of the folliculin (FLCN) gene was performed in four consenting patients from two families with Birt-Hogg-Dubé (BHD) syndrome, showing the occurrence of two frameshift mutations located respectively in exons 5 (802insA) and 9 (1345delAAAG) of the FLCN gene. A novel homozygous sequence variant in the intron 9 (IVS9 +5C>T) was also found. 1345delAAAG was associated with a wide variety of tumors, including stomach, colon, breast and parotid cancer. Conversely, the family carrying 802insA only had clinical evidence of dermatological lesions. These findings further suggest the relevance of exon 9 mutations in cancer predisposition for BHD.

Key words: Birt-Hogg-Dubé syndrome, cancer predisposition, molecular analysis, mutation, sequence variants

The Birt-Hogg-Dubé syndrome (BHD) (OMIM 135150) is a rare autosomal dominant genodermatosis predisposing patients to develop hair follicle hamartomas, lung cysts leading to pneumothorax, and an increased risk for renal neoplasia [1, 2]. The syndrome is caused by germline mutations in the folliculin (FLCN) gene (also known as BHD – OMIM 607273) which encodes a tumor-suppressor protein, FLCN. The latter, through its interaction with the FNIP1 protein, might thus represent a downstream effector of mTOR and AMPK and a modulator of energy/nutrient-sensing signaling pathways by some as-yet-unknown molecular mechanisms [3].
Numerous mutations have been described in the FLCN gene, the most frequent occurring within a C_8 tract of exon 11, resulting in a truncated folliculin; this "hot spot" mutation has been found in the germline of 44% BHD patients [4]. This hypermutability is probably due to a "slippage" in the DNA polymerase during DNA replication, resulting in gains or losses of repeat units, as happens for other genes causing cancer predisposition [5, 6]. However, recent reports suggest that all translated exons might be mutated [7, 8].
In the present study we describe the occurrence of two novel frameshift mutations of the FLCN gene in two Italian kindred affected by BHD.

Patients and methods

One four generation BHD kindred with 4 affected subjects (Family A) *(figure 1A)* and one three generation kindred with four affected subjects (Family B) *(figure 1B)* were studied. Genetic counseling was performed and family trees were obtained for both families.
Patients were referred to our Institutions following a request for dermatological medical advice. All of them underwent physical and instrumental examination. Clinically, affected BHD patients were diagnosed by the presence of 10 or more skin lesions clinically compatible with fibrofolliculomas, trichodiscomas or acrochordons. All consenting patients with BHD syndrome and their relatives underwent abdominal CT scanning and ultrasound screening for renal tumors and high-resolution CT scanning of the chest for air-filled lung cysts or granulomas. Of interest, no history of

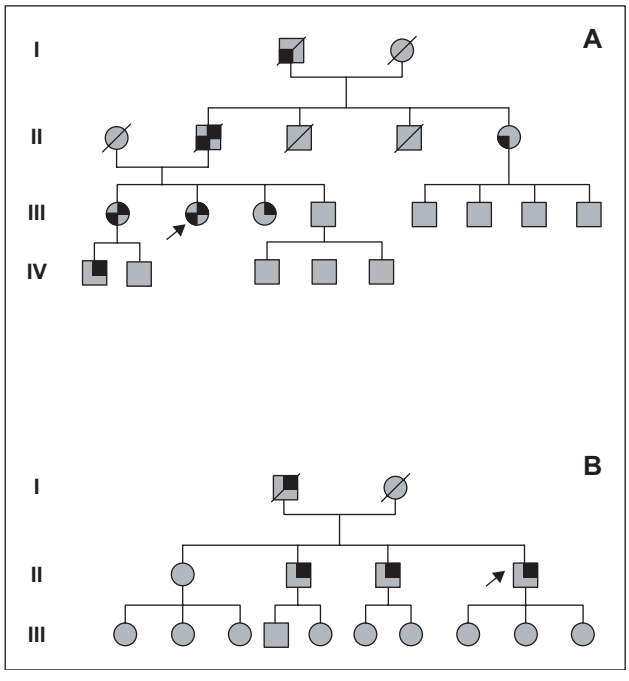

Figure 1. Pedigrees of the families studied. Arrow: proband. Solid top right quadrant: dermatological manifestations of Birt-Hogg-Dubè syndrome. Solid bottom left quadrant: cancer manifestation. Panel A: Family A, individuals III.1, III.2 and IV.2 were subjected to genetic testing. Panel B: Family B, proband II.4 was subjected to genetic testing.

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pneumothorax or renal tumor was recorded either in probands or their relatives. Histological biopsy examination was performed on consenting patients III.1, III.2, III.3 and IV.1 of Family A and patient II.4 of Family B *(table 1)*. The study was performed under the appropriate institutional ethics approvals and in accordance with the principles embodied in the Declaration of Helsinki. Written informed consent was obtained from each participating patient.

Molecular analysis of the FLCN gene was performed on genomic DNA isolated using QIAamp DNA Blood extraction kit (QIAGEN Inc., Chatsworth, CA) on peripheral blood leucocytes obtained from at least two independently drawn whole blood samples. The coding sequence and intron-exon borders of all 14 exons of FLCN (Gene ID: 201163) were amplified using the previously reported primer sequences and PCR conditions [4] and analyzed using single strand conformational polymorphism analysis [9] followed by sequencing. Sequencing reactions were performed using Big Dye Terminator (Applied Biosystem, Foster City, CA), and run on an ABI 3130 Genetic Analyzer (Applied Biosystem). All sequencing analyses were carried out, in order to exclude pre-analytical and analytical errors, on both strands and were repeated on PCR products obtained from new nucleic acid extractions. Furthermore, to better characterize the pathogenicity of the possible variants identified, 100 chromosomes from 50 individuals of the same ethnic background were also analyzed.

Results and discussion

The clinical and genetic features of BHD-affected individuals and their relatives are summarized in *table 1*. Family A *(figure 1A)* was a large four generation family from Central Italy. This family had 4 members with typical cutaneous fibrofolliculomas and was therefore considered as affected with BHD. The proband (III.2) was a 44 year old woman with long-standing and asymptomatic facial dome-shaped papules *(figure 2A)* that she had had for the last 20 years and a medical history of parotid pleiomorphic adenoma diagnosed 1-year before. Of the other three affected subjects the sister (III.1) had a history of breast cancer at the age of 44 and of colon cancer at the age of 56 while her 29 year old son (IV.1) and the other sister (III. 3) *(figure 2B)* showed only typical cutaneous fibrofolliculomas. The proband's father (II.2), referred skin lesions clinically compatible with fibrofolliculomas and with non UV-related squamous cell carcinoma of the lip at age 40, died at the age of 70 years of unknown causes. Other cancers detected in this family were a gastrointestinal cancer of colonic origin at the age of 50 in II.5 and an unspecific gastrointestinal cancer in I.1. In both cases no pathological data could be retrieved to ascertain the primary cancer site. As these relatives were deceased, it was not possible to perform mutational analysis and determine the mutation family co-segregation.

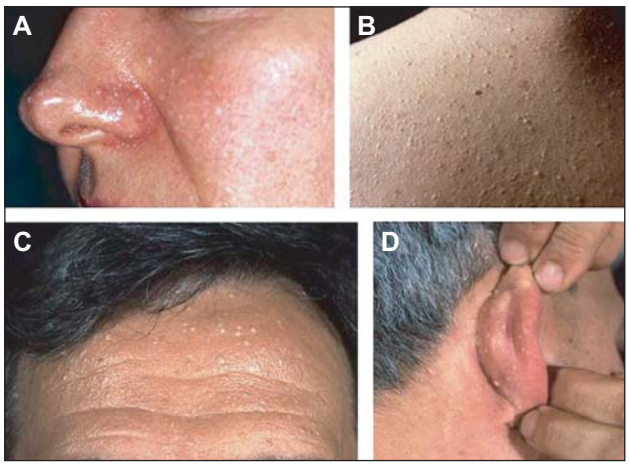

Figure 2. Skin manifestations in BHD patients. Multiple, asymptomatic papules **(A)** on the face of patient III.2 and **(B)** on superior back of patient III.3 of family A, **(C)** on the front of patient II.4 and **(D)** retroauricular region in patient II.2 from family B.

Family B *(figure 1B)* was a three generation BHD family from Central Italy. Five members were affected with typical fibrofolliculomas and therefore considered affected with BHD. The 50-year-old proband (II.4) had a history of dermatological lesions clinically compatible with BHD from the age of 30. Physical examination revealed multiple, asymptomatic, dome-shaped papules involving the face, neck, chest and back *(figure 2C)*. Diagnosis of fibrofolliculoma and trichodiscoma was histologically confirmed by biopsy *(figure 3)*. The two affected brothers (II.2, III.3), 57 and 60 years old respectively, showed only dermatological lesions, compatible with fibrofolliculomas *(figure 2D)*, while the father died at the age of 75 with referred signs of cutaneous lesions, similar to those of other family members. No history of other neoplasms was reported in the rest of the family.

Genetic testing was performed in all consenting relatives of Family A, including the proband's sister and her son (III.1 and IV.1). Sequencing of the FLCN gene revealed in the proband and her relatives an out-of-frame deletion of four bases (AAAG) starting at nucleotide 1345 of exon 9 that introduces a premature stop codon 24 aminoacids downstream in position 321 *(figure 4A)*. Conversely, an out-of-frame insertion of an adenine at nucleotide 802 of exon 5 that introduces a premature stop codon 16 aminoacids downstream in position 132 was found in the Family B proband *(figure 4B)*. In addition, a novel homozygous sequence variant of unknown biological significance in the intron 9 (IVS9 +5C>T) was found in the same patient.

Despite the similar ethnicity of the two BHD kindred, different mutations were identified in each family. In fact, mutational analysis showed the occurrence of two novel frameshift mutations, not previously reported, located respectively in exons 5 and 9 of the FLCN gene. Since genetic screening was conducted for diagnostic purposes, it was not possible to perform a co-segregation study in unaffected relatives of both families, who did not agree to undergo genetic analysis. This might represent a limitation to the study. However, in order to obtain further evidence regarding the potential pathogenetic role of the two novel identified mutations in exon 5 and 9 and to clarify the biological significance of IVS9+5 C>T, the three sequence variants were analyzed in 100 chromosomes from 50 unrelated control individuals of the same ethnic background. In this control population the 1345delAAAG in exon 9 and 802insA in exon 5 variants were absent in all individuals. Conversely, IVS9+5 C>T genotype frequencies were 63% C/T, 28% C/C and 9% T/T with a total frequency for the C

Table 1. Clinical and molecular characteristics of members of the two BHD families

Patient	Molecular analysis	Sex	Age (yrs) at examination	Skin lesions					Extracutaneous findings *(age of onset)*	Pulmonary/ Renal involvement	Histologic examination
				Type	Size	Colour	Localization	No			
Family A											
I1	NA	M	Deceased	-	-	-	-	-	Gastrointestinal cancer	NA	NA
II2	NA	M	Deceased 70	-	-	-	-	-	Squamous cancer of the lip (40)	NA	NA
II5	NA	F	Deceased 71	-	-	-	-	-	Gastrointestinal Cancer (50)	NA	NA
III1	1345delAAAG	F	58	Smooth papules	3-5 mm	Ivory-colored	Face	> 50	Breast Cancer (44) Colon Cancer (56)	NP	Fibrofolliculoma
III2	1345delAAAG	F	44	Domeshaped papules	3-5 mm	White	Nose-labial folds, face, neck and upperback	200	Parotid pleiomorphic adenoma (43)	NP	Fibrofolliculoma
III3	Consent denied	F	55	Domeshaped papules	2-3 mm	Skin colored	Upper back	> 30	Mental retardation	NP	Fibrofolliculoma
IV1	1345delAAAG	M	29	Smooth papules	2-3 mm	Ivory-colored	Face	> 10	NP	NP	Fibrofolliculoma
Family B											
I1	NA	M	Deceased 70	- *	-	-	-	-	NA	NA	NA
II2	Consent denied	M	57	Dome shape papules	3-4 mm	Skin colored	Front and retroauricolar	> 50	NP	NP	ND
II3	Consent denied	M	60	Translucide domeshaped papules	2-3 mm	Whitish	Face and nose	> 30	NP	NP	ND
II4	802insA IVS9+5C>T	M	54	Smooth papules	2-3 mm	White	Front and neck	> 50	NP	NP	Fibrofolliculoma Trichodiscoma

**Referred skin problems similar to those of other family members. No.: number; NA: not available; NP: not present; ND: not detected.*

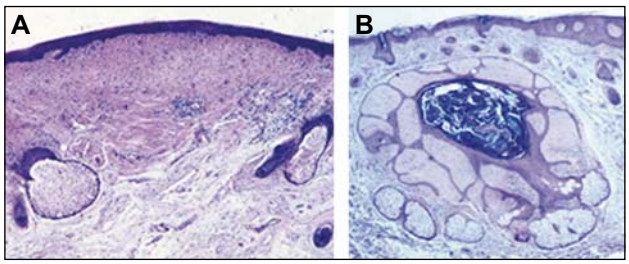

Figure 3. Biopsy of the papules of the patient II.4 of family B. **A)** Trichodiscoma: mit-like sebaceous lobules partially envelop a central zone composed of ribbon-like bundles of collagen, an increased number of fibrocytes and mucine. **B)** Fibrofolliculoma: abnormal hair follicle with epithelial strands extending from the infundibulum of the hair follicle into a hyperplastic mantle of specialized fibrous tissue.

and T alleles of 59 and 41%, respectively. These results indicated that IVS9+5 C>T represents a common polymorphism. To further support the non pathogenetic role of this variant, its influence on splicing mechanisms was also excluded using an on-line program at: http://www.fruitfly.org/seq_tools/splice.html.

On the other hand, the absence of both 1345delAAAG in exon 9 and 802insA in exon 5 variants in the control population and the predicted truncated protein *(figure 4)* strongly support the role of these mutations in the pathogenesis of BHD. Indeed, a recent study showed that FLCN interacts with FNIP1, a 130-kDa protein that is moderately conserved among species, suggesting that it serves an important function in living cells. This interaction occurs through its C-terminus *in vivo* and *in vitro*. Interestingly, nearly all mutations in BHD patients are predicted to produce a C-terminally truncated FLCN unable to bind FNIP1, and therefore compromised in its function the BHD phenotype [3].

Of interest, in the present study, Family B, carrying the 802insA mutation, showed isolated dermatological lesions, while, in Family A, the 1345delAAAG was associated with a wide variety of tumors, including gastrointestinal, parotid and, possibly, breast cancers. Given the early onset of breast cancer in III.1 and the lack of a positive family history, it was possible to hypothesize that breast cancer might be another component of the spectrum of tumors associated to BHD. However, given the high frequency of this cancer in the general female population, additional studies are needed to clarify this point. Moreover, the presence of colorectal cancer in III.1, and the clinical history of gastrointestinal cancer in two family members (I.1 and II.5), supports a relation of this malignancy with the BHD phenotype [5, 7, 12]. Of particular interest are the findings by Roggioletti *et al.* [12] who described a patient with BHD associated with intestinal polyps, one of which presented histological features of marked dysplasia. As the authors pointed out, this association may not be fortuitous and suggests that patients with multiple hamartomas of the perifollicular connective tissue should be examined periodically for intestinal polyps before malignancy develops [12].

Based on the present results and on literature data, the occurrence of BHD germline mutations different from the more common hotspot in exon 11 suggests the presence of different pathogenetic mechanism(s), which might be involved in tumor progression. Recently, it has been reported

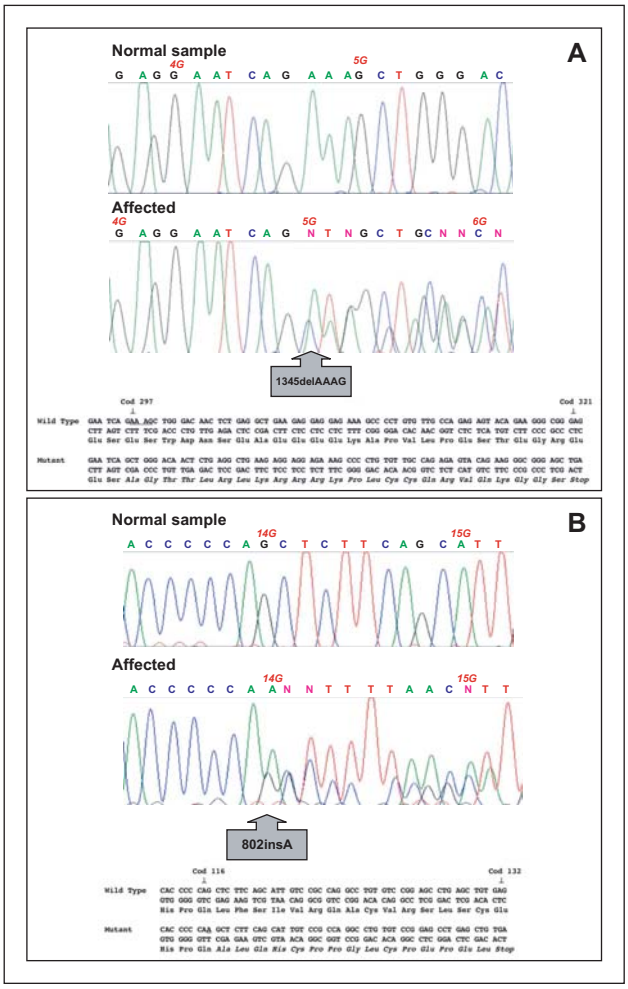

Figure 4. Sequence analysis of the BHD gene and predicted protein effect. Panel A: The affected proband of Family A showed an out-of-frame deletion of four bases (AAAG) at nucleotide 1345 of exon 9 (1345delAAAG). Panel B: The affected proband of Family B showed an out-of-frame insertion of an Adenine at nucleotide 802 of exon 5 (802insA). 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.

that a 4bp deletion in exon 4 seems to be associated only with primary spontaneous pneumothorax [11], whereas splice site mutations within intron 9 might be associated with a higher frequency of renal tumors [7]. Concerning this issue, Vocke *et al.* showed that renal tumors from a given BHD patient are associated with different somatic "second hits", thus supporting a role for BHD as a tumor suppressor gene predisposing to the development of renal tumors [13]. Although to date no correlation between the location of the BDH germline mutation and the nature of the "second hit" could be demonstrated, this possibility cannot be completely ruled out. Additional studies are warranted to further understand the mechanisms of BHD-induced tumorigenesis.

Nonetheless, the fact that a significant proportion of FLCN mutations are private should be taken into account in the mutational screening of the gene, and should lead to study

of the overall coding region [10]. While genotype-phenotype correlations remain to be elucidated for BHD, our findings demonstrate the occurrence of two novel germline mutations in the FLCN gene and might suggest the potential relevance of exon 9 mutations in cancer predisposition for BHD kindreds. ■

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