

Report

A 4-bp Deletion in the Birt-Hogg-Dubé Gene (*FLCN*) Causes Dominantly Inherited Spontaneous Pneumothorax

Jodie N. Painter,¹ Hanna Tapanainen,² Mirja Somer,⁴ Pentti Tukiainen,² and Kristiina Aittomäki^{1,3}

¹Folkhälsan Institute of Genetics, University of Helsinki, Departments of ²Pulmonary Medicine and ³Clinical Genetics, Helsinki University Central Hospital, and ⁴Department of Medical Genetics, Family Federation of Finland, Helsinki

Primary spontaneous pneumothorax (PSP), a condition in which air enters the pleural space and causes secondary lung collapse, is mostly sporadic but also occurs in families. The precise etiology of PSP remains unknown, although it is associated with emphysemalike changes (bullae) in the lungs of almost all patients. We describe the results of a genetic study of a large Finnish family with a dominantly inherited tendency to PSP. A genomewide scan suggested linkage to chromosome 17p11. Screening of the best candidate gene, *FLCN*, revealed a 4-bp deletion in the first coding exon, which causes a frameshift that predicts a protein truncation 50 missense amino acids downstream. All carriers of the deletion had bullous lung lesions. Mutations in *FLCN* are also responsible for Birt-Hogg-Dubé (BHD) syndrome (a dominantly inherited disease characterized by benign skin tumors, PSP, and diverse types of renal cancer) and, rarely, are detected in sporadic renal and colorectal tumors. Unlike other *FLCN* mutations, the exon 4 deletion seems to be associated with bullous lung changes only with 100% penetrance. These results suggest that changes in *FLCN* may have an important role in the development of PSP and, more importantly, of emphysema, a chronic pulmonary disease that often leads to formation of bullous lesions and lowered pulmonary function. Additionally, given the strong association of PSP and BHD, the connection between these conditions needs to be investigated further, particularly in patients with familial PSP, who may be at a greater risk of developing renal cancer.

Primary spontaneous pneumothorax (PSP [MIM 173600]) is a condition in which air is present in the pleural space in the absence of a precipitating event, such as trauma or lung disease. This results in secondary collapse of the lung, either partially or completely, and some degree of hypoxia (Sahn and Heffner 2000). PSP is relatively common, with an incidence between 7.4–18/100,000 for men and 1.2–6/100,000 for women (Melton et al. 1979; Bense et al. 1987*b*) and a dose-dependent, increased risk among smokers (Bense et al. 1987*a*). Most cases are sporadic, typically occurring in tall, thin men aged 10–30 years (Primrose 1984) and generally while at rest. Familial PSP is rarer and usually is inherited as an autosomal dominant condition with reduced penetrance (Abolnik et al. 1991; Morrison et al. 1998; Bagchi

and Nycyk 2002), although X-linked recessive (Abolnik et al. 1991) and autosomal recessive (Koivisto and Mustonen 2001) inheritance have also been suggested. The cause of most familial cases is unknown, although PSP may occur in inherited connective tissue disorders, such as Marfan (Dwyer and Troncale 1965) and Ehlers-Danlos (Dowton et al. 1996) syndromes and may be associated with particular human leukocyte antigens (Sharpe et al. 1980; Yamada et al. 2003) and alpha 1-antitrypsin deficiency (Daniel and Teba 2000).

Although PSP is said to occur without underlying lung disease, almost all patients have subpleural blebs or bullae in their lungs, detectable by computed tomography, thoracoscopy, or thoracotomy (Schramel et al. 1997). Bullae, or localized emphysemalike changes (ELCs), are thin-walled air spaces >1 cm in diameter, distal to terminal bronchioles, and associated with lung-tissue destruction. The development of ELCs is associated with degradation of the elastic fibers of the lung (Fukuda et al. 1994) and is thought to involve complex interactions between inflammatory processes, protease-antiprotease and oxidant-antioxidant imbalances, matrix remodeling, and mechanical forces (Suki et al. 2003).

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Address for correspondence and reprints: Dr. Jodie Painter, Folkhälsan Institute of Genetics, Biomedicum Helsinki, P.O. Box 63, University of Helsinki, FIN-00014, Helsinki, Finland. E-mail: jodie.painter@helsinki.fi

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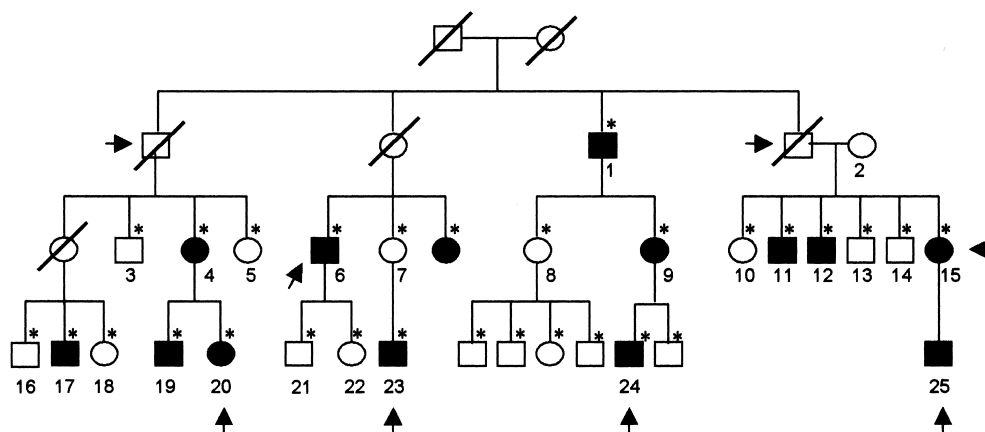


Figure 1 Simplified pedigree of the Finnish family with PSP. HRCT (indicated by an asterisk [*]) was performed for all living family members except individual 25. Affected members (blackened symbols) have ≥ 1 bullae on their lungs, detected by HRCT or during surgery (individual 25). Arrows indicate those who had experienced a PSP episode, and numbers indicate those from whom DNA samples were obtained. The presence of bullae could not be confirmed for two individuals who died prior to the study; hence, they were classified as unaffected, even though both had experienced PSP.

We recently identified a large Finnish pedigree with a tendency to PSP, which appears to be dominantly inherited (fig. 1). Here, we report the results of a genomewide scan and the identification of a deletion in the folliculin gene (*FLCN* [MIM 607273; GenBank accession number NM_144997]), also responsible for Birt-Hogg-Dubé syndrome (BHD [MIM 135150]). The family was identified through the index patient (individual 15), who had been referred to the Department of Clinical Genetics at the Helsinki University Central Hospital to be evaluated for a possible connective-tissue disorder. Clinical examinations (conducted independently by K.A. and M.S.) revealed no clinical signs or symptoms of either Marfan syndrome or Ehlers-Danlos syndrome. Alpha 1-antitrypsin deficiency was also excluded. This woman had experienced PSP twice, and her son (individual 25) once. Both individuals had numerous bullae on their lungs (fig. 2). Subsequently, the lungs of 28 additional family members were examined with high-resolution computed tomography (HRCT). Twelve of these family members had between 1 and >30 bullae, 1–6 cm in diameter, randomly situated in the lungs. All individuals with bullae (14 in total) were classified as “affected.” One female (individual 7), whose son (individual 23) had bullae and had experienced a PSP episode, had a normal HRCT. PSP history was determined by interviewing family members and was confirmed from hospital records, when available. In total, eight members (including two who had died prior to the study) had each had between one and four PSP episodes. Informed consent was obtained from all participating family members, and the study was approved by the institutional ethics committee.

Genomic DNA was extracted from blood samples of 25 family members, 13 affected and 12 unaffected. The

genome-scan genotyping and scoring was performed by the Finnish Genome Center, University of Helsinki, by use of the Linkage Mapping Set MD10 (Applied Biosystems), with an average intermarker distance of 10 cM. Genotypes were obtained for 371 markers. Two-point LOD scores were calculated with the MLINK program of the FASTLINK package (Cottingham et al. 1993), and multipoint and two-point nonparametric linkage (NPL) scores were calculated with GENEHUNTER 2.0 (Kruglyak et al. 1996). For all analyses, inheritance was assumed to be autosomal dominant with reduced penetrance (85%), with a disease allele frequency of 0.001. Allele frequencies for each marker were the same as for the Finnish population (Finnish Genome Center).

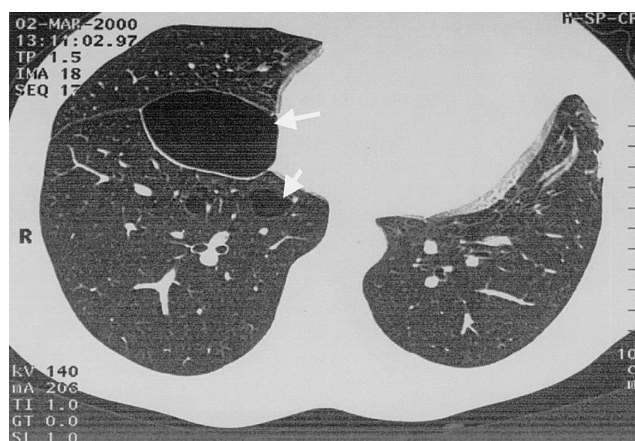


Figure 2 HRCT scan of the index patient, who experienced recurrent episodes of PSP. Numerous bullae are visible, seen as clear, black areas. The two largest bullae are indicated with arrows.

Evidence of linkage was observed on chromosome 17p11, with single-point linkage peaks at markers *D17S1852* (NPL = 2.31; $P = .006$) and *D17S798* (LOD = 3.43 at recombination fraction $[\theta] = 0.001$). Haplotype analysis defined the region shared by all affected family members to ~34 cM between these two markers. The marker closest to the multipoint linkage peak (NPL = 13.45; $P = .0009$) was *D17S1857*. Located between *D17S1857* and *D17S798* is the *FLCN* gene, responsible for BHD. This gene was a very good positional candidate because of the 50-fold increased risk of PSP in patients with BHD (Zbar et al. 2002). Primers for the amplification and sequencing of the 14 exons were as detailed by Nickerson et al. (2002).

We found a 4-bp deletion (c.733delTCGG) in exon 4, the first coding exon, of *FLCN* (fig. 3). (Note that this numbering is in reference to GenBank mRNA sequence NM_144997.) Previous mutations have been annotated in reference to GenBank sequence AF517523, which contains 43 fewer bases of 5' UTR sequence (the equivalent base in the AF517523 mRNA sequence is nt 690). The 4-bp deletion would cause a frameshift and would result in a TGA termination codon 50 missense amino acids downstream. All family members with bullous lung lesions were heterozygous for this deletion, with the exception of one male (individual 23), who had experienced one PSP episode. Although his mother (individual 7) was a suspected PSP carrier, her normal HRCT result indicated that she did not have the deletion. The apical location of his bullae (bullae typically occur near the lung apex in sporadic cases but are situated randomly in familial ones) strongly suggests that individual 23 experienced PSP as a sporadic event and not through inherited susceptibility.

The deletion was not present in unaffected family members or in control samples comprising 100 healthy Finnish individuals and a subgroup of 50 individuals born in the Savo region, an area of early settlement for the Finnish population and the birthplace of the grandparents of the index subject (controls were screened by denaturing high-performance liquid chromatography with a 3500HT WAVE [Transgenomic]). The absence of the deletion in unaffected family members indicates that the penetrance of this mutation in this family is extremely high—apparently 100%—when the presence or absence of bullae is considered as the affection status. Generally, affection status in families with PSP is determined by pneumothorax episodes; hence, penetrance has been suggested to be fairly low, 50% for males and 34% for females (Abolnik et al. 1991). Indeed, had we considered only PSP episodes, penetrance estimates for this family would have been similarly low, particularly for females (two of eight cases). Our results highlight the particular importance of consideration of bullous lung changes in familial PSP.

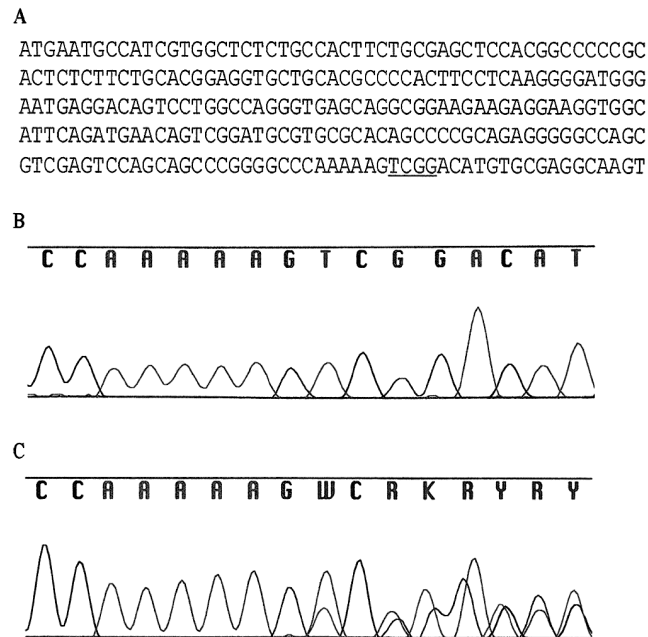


Figure 3 A 4-bp deletion in exon 4 of the *FLCN* gene. A, Exon 4 sequence. The deletion is underlined. B, Unaffected control sequence. C, Affected sequence, showing the wild-type and c.733delTCGG alleles.

To date, most of the reported mutations in *FLCN* cause BHD syndrome (Birt et al. 1977), a dominantly inherited condition characterized by multiple, benign tumors of the hair follicle (fibrofolliculomas) that typically appear as pale or skin-colored papules over the face, neck, and upper trunk at age ~20–30 years. In addition, 70% of patients with BHD have pulmonary cysts, 25% experience PSP, and 15%–30% develop diverse types of renal cancer (Choyke et al. 2003). Other manifestations, particularly colorectal tumors (Khoo et al. 2002), have been reported but are not considered to be part of the BHD phenotype (Zbar et al. 2002; Vincent et al. 2003). More rarely, somatic mutations, loss of heterozygosity, and methylation of the promoter have been detected in diverse types of sporadic renal and colorectal tumors (Khoo et al. 2002, 2003; da Silva et al. 2003; Shin et al. 2003; Nagy et al. 2004), consistent with the Knudson two-hit model (Knudson 1971), which highlights a potential role for *FLCN* as a tumor-suppressor gene.

The Finnish family under investigation appears to experience only PSP—there are no obvious indications of fibrofolliculomas (or other benign skin tumors) or renal cancer in any member. There are a number of possible explanations for this. The absence of BHD symptoms may be due to the location of the deletion, which occurs in the first coding exon. Whereas almost all mutations in *FLCN* reported to date are truncating, most BHD- and cancer-associated mutations, with the exception of

one somatic exon 4 missense mutation in one colorectal carcinoma (Kahnoski et al. 2003), occur downstream in the gene. Of familial and sporadic BHD mutations, ~50% are due to indels in a mutation hotspot in a C₈ tract in exon 11. Alternatively, given that less than half of all patients with BHD develop renal cancer and that *FLCN* mutations are found in only a small number of sporadic renal and colorectal tumors, the cancer phenotype may be determined by other modifier genes that are absent from this particular family or from the Finnish population.

It is also possible that the deletion does, in fact, cause BHD in this family and that either the BHD phenotype is very mild and atypical, in that PSP is the predominant manifestation, or the onset of the characteristic skin manifestation is delayed. Fibrofolliculomas typically develop in patients with BHD by age 40 years. Prior to this age, patients are more likely to experience PSP than are older patients, whereas those aged >40 years, particularly affected men, are more likely to develop renal cancers (Zbar et al. 2002) than are younger patients.

The affected members of the family with PSP currently have an age range of 21–83 years (average age 49 years), and all but three affected members are aged ≥44 years. Since this family was initially ascertained as suffering from PSP, the members were not screened for specific BHD manifestations. However, prior to the discovery of the deletion, several family members were independently examined by experienced clinical geneticists (K.A. and M.S.) for the possibility of an unknown syndrome. Skin was examined particularly closely for signs of Ehlers-Danlos syndrome (for which the genotype is skin that is variably thin, hyperextensible, and prone to bruising). Because of the close association of PSP and BHD, Zbar et al. (2002) suggested that the BHD gene, then unidentified, might be responsible for some cases of familial PSP and that PSP could be used as a diagnostic criterion for BHD in members of families with BHD. Our results highlight the need to also consider the possibility of BHD in families with PSP, particularly from a clinical perspective, since these patients may be at greater risk of developing renal cancer. We are currently organizing genetic counseling and appropriate examinations, including abdominal scans, for consenting family members.

At this point, we can only speculate as to the potential role of *FLCN* in PSP. The function of the *FLCN* protein is currently unknown, and it has no known human homologs, although the protein is highly conserved across species (Nickerson et al. 2002). In two animal models, German Shepherd dogs (Lingaas et al. 2003) and the Nihon rat (Okimoto et al. 2004), heterozygous mutations cause renal cancer with apparently complete penetrance, and there is evidence of a homozygous lethal effect. In humans, *FLCN* mRNA is selectively expressed in specific cell types in a number of tissues, including skin, lung,

kidney, pancreas, breast, prostate, and cerebellum, which suggests roles in secretion or endocytosis/phagocytosis, in addition to tumor suppression (Warren et al. 2004). A role in secretion is supported by the findings (1) of Nickerson et al. (2002), which suggest a defect in the interaction between epithelial and mesenchymal cells in fibrofolliculomas of patients with BHD, and (2) of Shin et al. (2003), which suggest that cellular proliferation in colorectal cancer is affected by alteration of the extracellular matrix composition.

The precise pathogenesis of PSP is also unclear. Bullae are associated with PSP but may be more indicative than causative of underlying changes to the lungs. Whether bullae are the site of rupture—and whether they have predictive value for the occurrence and recurrence of PSP—are the subjects of considerable debate (see Schramel and Zanen [2001]). The development of both bullae and PSP is, however, associated with degradation of elastic fibers in the lung. In emphysema, this degradation is initiated by a protease-antiprotease and an oxidant-antioxidant imbalance that result from an influx of inflammatory cells, particularly macrophages (Barnes 2000). The role of inflammation in the initiation of ELCs is supported by evidence from smokers, who have increased numbers of bronchial macrophages in addition to a 9-fold increased risk among women and a 22-fold increased risk among men of developing pneumothorax (Bense et al. 1987a).

Within the lung, *FLCN* mRNA is expressed most strongly in the stromal cells (macrophages and fibroblasts) of the connective tissue and in macrophages within the alveolar space and is expressed moderately in type I pneumocytes, the epithelial cells lining alveoli (Warren et al. 2004). This expression pattern suggests that the effect of the mutation in lungs may be mediated, particularly through macrophages and possibly through fibroblasts. Both cell types variously secrete a complex mix of factors—including cytokines, chemokines, and proteases—and an imbalance may either induce an inflammatory response even in the absence of an obvious trigger (such as cigarette smoke) or alter matrix degradation and remodeling.

In this Finnish family, a 4-bp deletion in *FLCN* causes inherited PSP. However, determination of the general importance of *FLCN* in the development of PSP requires further screening of both familial and sporadic patients for mutations in this gene. This, in turn, may help to elucidate the function of the *FLCN* protein in the lung, in association with PSP and, even more importantly, with generalized emphysema, and perhaps may shed further light on its role in tumorigenesis.

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Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

GenBank, <http://www.ncbi.nlm.nih.gov/Genbank/> (for *FLCN* [accession numbers NM_144997 and AF517523])
Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for PSP, *FLCN*, and BHD)

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