Lung
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Genetic Analysis of Familial Spontaneous Pneumothorax in an Indian Family

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Abstract Familial spontaneous pneumothorax is one of the phenotypes of Birt–Hogg–Dubé syndrome (BHDS), an autosomal dominant condition associated with folliculin (*FLCN*). We investigated clinical and genetic data of an Indian family having two patients suffering from spontaneous pneumothorax in the absence of skin lesions or renal tumors. HRCT scan of patient's lung revealed paracardiac cysts, and DNA sequencing of all 14 exons of *FLCN* from patients showed the presence of heterozygous "*C* allele" deletion in the poly-cytosine (poly-*C*) tract of exon 11 leading to truncated folliculin. This mutation was also observed in four asymptomatic members of the family. Our results confirmed the presence of deletion mutation in poly-*C* tract of *FLCN* in members of BHDS family. This is the first report of genetic insight in a BHDS family from India but in-depth studies with a larger sample set are necessary to understand mechanism of familial pneumothorax.

Keywords Birt–Hogg–Dubé syndrome · Spontaneous pneumothorax · Lung cysts · Folliculin · Genetic analysis · Indian family

Abbreviations

HRCT	High-resolution CT
BHDS	Birt–Hogg–Dubé syndrome
FLCN	Folliculin
c.+1285	Nucleotide position (np) in cDNA sequence

Introduction

Spontaneous pneumothorax is an abnormal collection of air in pleural space that separates lung from thoracic wall. Most individuals suffering from this disease have no prior history of lung disease though smoking habit and trauma can be predisposition factors [1, 2]. CT scans of these individuals may show lung cysts or sub-pleural blebs in the lung which can be characterized as primary spontaneous pneumothorax [3]. Familial spontaneous pneumothorax, first reported in 1921 [4] follows an autosomal dominant pattern of inheritance with incomplete penetrance. Some other genetic disorders linked to spontaneous pneumothorax are alpha 1-antitrypsin deficiency, cystic fibrosis, Marfan syndrome, Ehlers–Danlos syndrome, tuberous sclerosis, and BHDS (MIM 135150) [4, 5]. However, most of the reported cases of BHDS are from western countries and diagnosis of this condition in Asian population is considerably low. Considered as a monogenic disorder this has been mapped to a gene, *FLCN*, [genbank accession no. AF517523], in chromosome 17p11.2 [6]. *FLCN* is composed of 14 exons and an indel mutation of cytosine in hypermutable poly-*C* tract (C_8) in exon 11 has been detected in ~53 % of the BHDS families [7, 8]. This germline mutation is predicted to cause a frameshift and truncation of folliculin protein which is known to express in a number of tissues like stromal cells, distal nephron of the kidney, type I pneumocytes of lung [9, 10]. It is also

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known to play a key role in important metabolic pathways but how it contributes to cyst development in BHDS is still unknown.

We examined a family with spontaneous pneumothorax for genetic mutations in *FLCN* to study correlation between genetic data and their phenotypes. The mutation data were then compared with unrelated healthy controls.

Methods

This study was approved by "Review committee for protection of research risk to humans, Indian Statistical Institute." A three generation Indian family (Fig. 1), with two siblings suffering from spontaneous pneumothorax was recruited for this study. Ages of the parents (father-65 years old, mother-60 years old), siblings and index patients (range from 27 to 39 years) and third generation boy (13 years) were noted during recruitment in this study. Examination of the patients eliminated skin folliculomas and renal tumors by thorough dermatologic examination and abdominal CT, respectively. Patients were evaluated by spirometry and for concentrations of alpha-1-anyitrpsin. Non-contrast high-resolution CT scan of chest of 4 members (two with pneumothorax, patients II-4 and II-5, and two unaffected, II-6 and III-1) from the family were taken with a 16-slice CT scanner (Brivo CT385; GE Healthcare), where the patients were examined in supine position with breath holding in deep inspiration from apices of lung to costophrenic angles. CT scan of other family members was not possible due to logistic problem and unwillingness of the family members. For genetic analysis, genomic DNA was isolated using the QIAamp Blood extraction kit (QIAGEN Inc., Valencia, CA) from venous blood ($\sim$2 ml) of 8 of the 10 members of the family and 14 unrelated healthy controls. Two members of the family, sister (II-7), and husband (II-3) of index patient did not participate in the study. Bidirectional sequencing of PCR products were performed in ABI 3100 Genetic Analyzer (ABI Inc., USA) using primers (Supplementary Table 1) designed specifically to include all the 14 exons and their boundaries. Reference alignments and variation calling was done using two sequence alignment softwares (BioEdit and Seqman).

Results

History and Clinical Features

Index patient's (II-4) father (I-1) and mother (I-2) had none of the clinical manifestations of Birt–Hogg–Dubé syndrome (Fig. 1). Second generation consists of 6 siblings

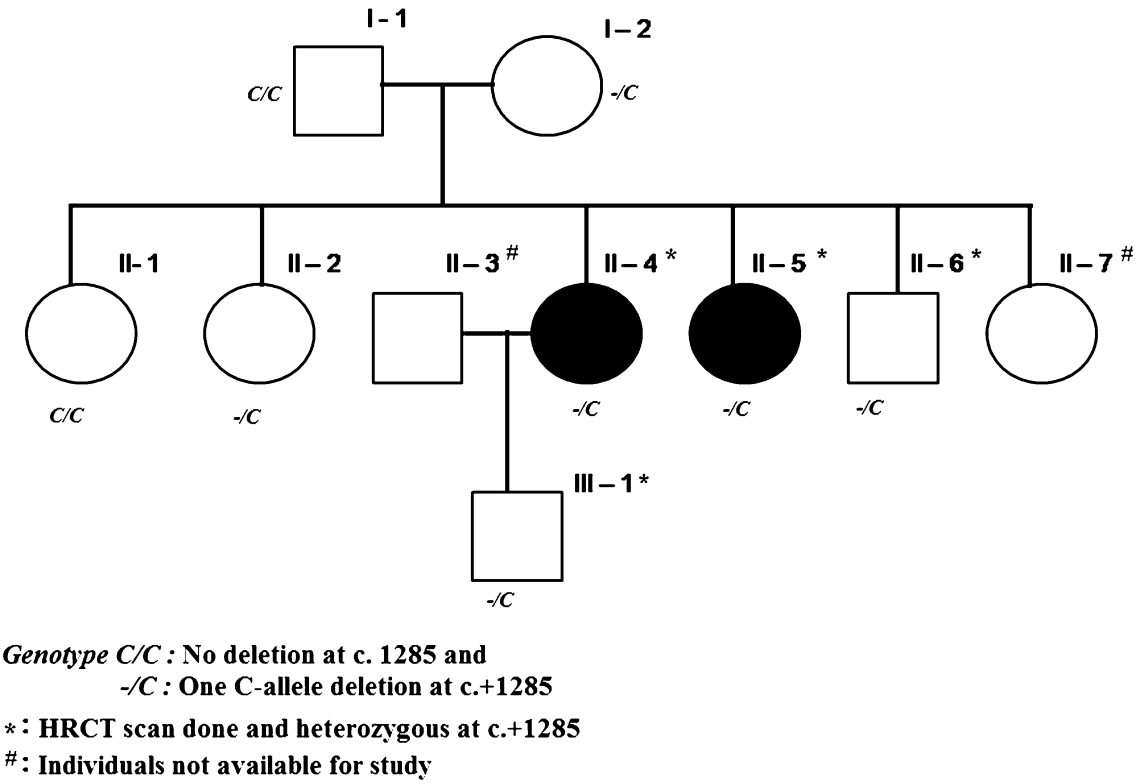

Fig. 1 Pedigree of the patient family, *Circles*: females, *Squares*: males; *open symbols*: unaffected individuals, *closed symbols*: BHDS patients. Generations are identified by *Roman numerals* and individuals with *Arabic numbers*. Index patient: II-4

Table 1 Polymorphisms found in *FLCN* in patient family members and healthy controls

Patient family member	Exon 1 C > T rs1708629	IVS3[a] C > G rs1736212	IVS5 C > T rs1736219	IVS9 C > T rs8065832	IVS10[b] (A > C)	Exon 11 c.+1285 *C* del p.429His > Thr	IVS11 C > T rs34311146	IVS12 A > G rs34235236	Exon 14 T > C rs3803761	Disease status
I-1	*TT*	*GG*	*TT*	*TT*	*AC*	*CC*	*CT*	*AA*	*CC*	Unaffected
I-2	*TT*	*GG*	*TT*	*TT*	*AC*	*c.1285Cdel (−/C)*	*CC*	*AG*	*CC*	Unaffected
II-1	*TT*	*GG*	*TT*	*TT*	*AA*	*CC*	*CC*	*AG*	*CC*	Unaffected
II-2	*TT*	*GG*	*TT*	*TT*	*AA*	*c.1285Cdel (−/C)*	*CT*	*AA*	*CC*	Unaffected
II-4	*TT*	*GG*	*TT*	*TT*	*AC*	*c.1285Cdel (−/C)*	*CC*	*AA*	*CC*	*Pneumothorax*
II-5	*TT*	*GG*	*TT*	*TT*	*AC*	*c.1285Cdel (−/C)*	*CC*	*AG*	*CC*	*Pneumothorax*
II-6	*TT*	*GG*	*TT*	*TT*	*AC*	*c.1285Cdel (−/C)*	*CC*	*AA*	*CC*	Unaffected with cysts
III-1	*CT*	*CG*	*CT*	*CT*	*AC*	*c.1285Cdel (−/C)*	*CC*	*AA*	*TC*	Unaffected
Healthy control group										
C-1	*CT*	*CC*	*CT*	*CT*	*AC*	*CC*	*CC*	*AA*	*CC*	Control
C-2	*TT*	*GG*	*CC*	*CC*	*AC*	*CC*	*CT*	*AG*	*CC*	Control
C-3	*CT*	*CG*	*CC*	*CT*	*AC*	*CC*	*CC*	*AA*	*TC*	Control
C-4	*CC*	*GG*	*TT*	*TT*	*AC*	*CC*	*CC*	*AA*	*CC*	Control
C-5	*TT*	*GG*	*TT*	*TT*	*AC*	*CC*	*CC*	*AA*	*CC*	Control
C-6	*CC*	*CC*	*TT*	*CC*	*AC*	*CC*	*CC*	*AA*	*TT*	Control
C-7	*CT*	*CG*	*CT*	*CT*	*AC*	*CC*	*CT*	*AA*	*CC*	Control
C-8	*TT*	*CC*	*TT*	*TT*	*AC*	*CC*	*CC*	*AA*	*CC*	Control
C-9	*CT*	*CC*	*CT*	*CT*	*AC*	*CC*	*CC*	*AA*	*CC*	Control
C-10	*CT*	*CG*	*CT*	*TT*	*AC*	*CC*	*CT*	*AA*	*TC*	Control
C-11	*CT*	*GG*	*CT*	*CT*	*AC*	*CC*	*CC*	*AA*	*CC*	Control
C-12	*CC*	*GG*	*TT*	*TT*	*AC*	*CC*	*CC*	*AA*	*CC*	Control
C-13	*CC*	*CG*	*CC*	*CT*	*AC*	*CC*	*CC*	*AA*	*CC*	Control
C-14	*CC*	*CG*	*CC*	*CC*	*AC*	*CC*	*CC*	*AA*	*TT*	Control

[a] *IVS* intronic region, *c.+1285* nucleotide position in cDNA (according to HGVS nomenclature)

[b] IVS 10 A > C, is a novel SNP in this population at genomic location-chr17:17,217,007(GRCh38/hg38 assembly)

and two of them are affected members (II-4, II-5). The index patient (II-4), 35-year-old female, non-smoker, was suffering from acute shortness of breath and left-sided chest pain. Medical history, clinical examination, and chest X-ray confirmed diagnosis of left-sided pneumothorax. Intercostal tube drainage improved her symptomatically. CT scan of her chest (Fig. 2a) showed diffusely scattered lung cysts of varied sizes in well-aerated right lung with huge bulla in between inter-lobular fissure with some paraspetal emphysematous bullae as well as some on left side and a collapse of left lower lobe retracted toward hilum. Second symptomatic individual (II-5), 29-year-old female has a history of recurrent left-sided spontaneous pneumothorax which needed hospitalization 2 years back, where she underwent chemical pleurodesis with 10 % aqueous povidoneiodine. CT scan (Fig. 2b) of this patient revealed multiple lung cysts in both lungs with one large cyst adjacent to pericardium on left lower lobe. Brother (II-6) of the patient, 27 year-old and non-smoker, did not suffer from emphysema or pneumothorax, but had few well-defined cysts (Fig. 2c) in the paracardiac region (i.e., lung characteristics of BHDS) with normal renal shadows. The index patient has a 13-year-old son (III-1) with no medical manifestations of BHDS and the kid presented a clean CT (Fig. 2d) on radiological examination. None of the affected members tested positive for alpha-1 antitrypsin deficiency, Marfan, or Ehlers–Danlos syndrome.

Mutation Analysis

Sequencing analysis revealed a heterozygous *C* deletion in the poly-*C* tract in Exon 11 (Supplementary Fig. 1a and b), cDNA nucleotide position (c.+1285), leading to a premature termination of FLCN protein due to generation of a new stop codon at c.+1401, instead of original c.+1720 in isoform 1 of *FLCN* transcript in six members of the three generation family (Fig. 1). Members II-4 and II-5 suffered from pneumothorax, while four other members, not suffering from pneumothorax, were two other siblings, II-2, II-6, mother (I-2) and grandson (III-1). Mutation was absent in father (I-1) and a female sibling (II-1) (Supplementary Fig. 1c) of the index patient. However, CT investigations, of two asymptomatic individuals harboring heterozygous *C* deletion, revealed few well-defined cysts in a member, II-6, but no cysts or air pockets in the lung of grandson (III-1). However, this heterozygous *C* deletion was not found in any of the healthy control individuals (Table 1). A number of other SNPs, present in both patient family members and control individuals, were also identified in intronic and UTR non-coding regions of *FLCN* (Supplementary Fig. 2; Table 1).

Discussion

After sequencing, nine SNPs were found in *FLCN* (Supplementary Fig. 2) and eight of them have been reported previously [11, 12] but IVS 10 A > C, is a novel SNP in this population at genomic location—chr17:17,217,007(GRCh38/hg38 assembly). Except the heterozygous *C* deletion in exon 11, all family members and unrelated healthy controls shared the same genotype at 8 SNP loci (Table 1) indicating that *C* deletion to be strongly associated with BHDS. Polymerase slippage during DNA replication can be a cause of *C* nucleotide deletion in the hypermutable site, poly-C_8 tract [13]. Presence of heterozygous *C* deletion divides the family members into two groups with respect to symptomatic conditions. First group contains two individuals who suffered from pneumothorax and harbor the *C*-allele deletion, and the second group has four individuals with no incidence of pneumothorax or lung distress but harbor *C*-allele deletion. Like the individuals of second group, there is report for the presence of *C*-allele deletion without any lung distress [7]. But pulmonary cysts were found in the basal region of lung in asymptomatic individuals (Fig. 2c) unlike primary pneumothorax which have apical lung cysts, therefore, suggesting the presence of Birt–Hogg–Dubé syndrome in this family [14]. However, 13-year-old son of index patient presented a clean CT image, which perhaps explains adult age for onset of clinical manifestations although there are recent reports of pneumothorax and lung cysts in pediatric patients carrying the *FLCN* mutation [15, 16]. Therefore, the child needs evaluation for any pulmonary distress or renal anomalies regularly, as he has a risk for the syndrome. Smoking habits as a risk for spontaneous pneumothorax [17] were ruled out in this study since all members were non-smokers in this family. Here, *C* allele deletion mutation was detected in six members of the family but the phenotype was manifested in only two patients; therefore, it may be postulated that a single deletion might not be sufficient to cause any of BHDS manifestations. Perhaps, there lies a strong involvement of other genetic factors that lead to the conditions. The *C*-allele deletion at c.+1285 of *FLCN* causes a frame shift mutation leading to a truncated protein with 466 amino acids instead of 579 due to the formation of a new termination codon at c.+1401 (Supplementary Fig. 3). We analyzed the reference and mutated protein sequences in silico, where secondary structure predicted presence of a single pair beta-hydrogen bond instead of an alpha helix at the 466th residue, creation of a different frames of residues from codon 429 ($H > T$) till termination and altered 3-D structure (data not shown).

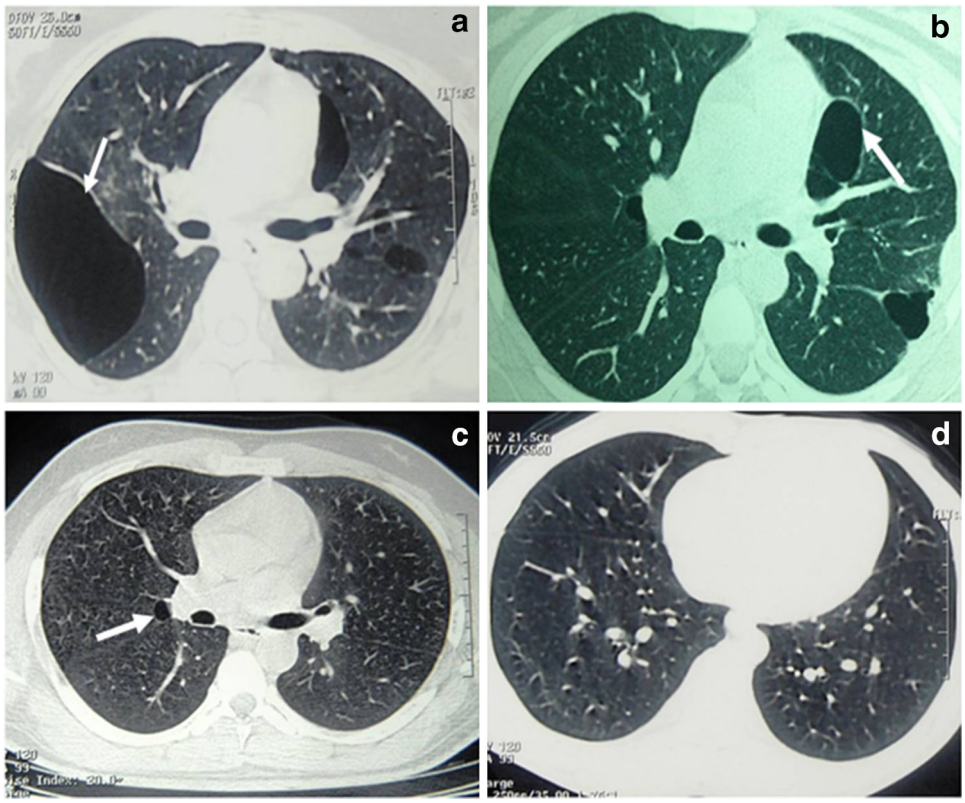

Fig. 2 High-resolution CT scans of patients and family members. **a** and **b** Patients (II-4) and (II-5) (see Fig. 1), both having episodes of spontaneous pneumothorax. **c** Brother (II-6) of patients showing presence of paracardiac cysts and **d** no cysts or air pockets in lungs of grandson (III-1) in family. *White arrows* in **a**, **b**, and **c** indicate the presence of lung cysts

Folliculin plays important roles in key metabolic pathways—such as *mTOR* and *AMPK* [18]; a truncated *FLCN* may lead to loss of epithelial cells due to apoptosis thus contributing to altered lung function in BHDS. A recent study suggests a model, where the mutated *FLCN* downregulates membrane localization of *E-cadherin* and *LKB1*, impairing *AMPK* activation which in turn hinders cell survival resulting in increased surface tension, alveolar collapse, and impaired pulmonary function [19]. However, another study shows that loss of *FLCN* results in constitutive activation of AMPK which induces autophagy and inhibits apoptosis improving cellular bioenergetics in renal neoplasia [20]. How the *C*-allele deletion in exon 11 of *FLCN* (i.e., truncated protein) is involved in two different kinds of manifestations (i.e., cancer and pneumothorax) is puzzling. In cancer, loss of apoptosis is observed, whereas in pneumothorax apoptosis leads to loss of epithelial cells leading to lung collapse. Only comprehensive molecular and functional insights with a larger sample set can help solve this riddle.

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Conflict of interest The authors declare that they have no conflict of interest.

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