Novel mutations in the folliculin gene associated with spontaneous pneumothorax

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ABSTRACT: Spontaneous pneumothorax is mostly sporadic but may also occur in families with genetic disorders, such as Birt–Hogg–Dubé syndrome, which is caused by mutations in the folliculin (*FLCN*) gene.

The aim of the present study was to investigate the presence and type of mutation in a Swiss pedigree and in a sporadic case. Clinical examination, lung function tests and high-resolution computed tomography were performed. All coding exons and flanking intronic regions of *FLCN* were amplified by PCR and directly sequenced. The amount of *FLCN* transcripts was determined by quantitative real-time RT-PCR.

Two novel mutations in *FLCN* were identified. Three investigated family members with a history of at least one spontaneous pneumothorax were heterozygous for a single nucleotide substitution (c.779G>A) that leads to a premature stop codon (p.W260X). Quantitative real-time RT-PCR revealed a reduction of *FLCN* transcripts from the patient compared with an unaffected family member. DNA from the sporadic case carried a heterozygous missense mutation (c.394G>A). Lung function of this patient was normal and computed tomography showed similar bilateral cysts, as observed in the two members of the unrelated Swiss family.

Mutations in the folliculin gene are associated with cystic lung lesions in an otherwise morphological normal lung and predispose to spontaneous pneumothorax.

KEYWORDS: Familial pneumothorax, folliculin gene, mutation, pneumothorax

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spontaneous pneumothorax is a collection of air in the pleural space of the lung, causing the lung to collapse. A majority of individuals with a spontaneous pneumothorax have no obvious lung disease (''primary'' spontaneous pneumothorax), but intraoperative inspection or preoperative computed tomography (CT) scans generally reveal the presence of small subpleural blebs of the lung [1, 2]. The pathogenesis of these subpleural blebs is probably related to airway inflammation secondary, to some extent, to cigarette smoking in many cases. Pneumothorax also occurs with increased rate in patients suffering from hereditary connective tissue disorders, such as Marfan syndrome and Ehlers–Danlos syndrome [3, 4]. The clustering of pneumothorax events in families not affected by a connective tissue disease is well known, and autosomal dominant, autosomal recessive, X-linked recessive, as well as polygenic, inheritances have been suggested [5–7]. The autosomal dominant Birt–Hogg–Dubé syndrome (BHDS) is a genodermatosis predisposing patients to benign skin tumours

and renal cancer. It is also associated with an increased incidence of spontaneous pneumothorax [8, 9]. In a study of 198 patients with BHDS, pneumothorax occurred in 24% of the cases [10]. It has been shown that mutations in the folliculin (*FLCN*) gene on chromosome 17p11.2 can cause this syndrome [11]. Other mutations in *FLCN* have been associated with spontaneous pneumothorax and bullous lung disease in the absence of the oncological manifestations of BHDS [12, 13].

The current authors have recently studied a Swiss pedigree, with several members who had experienced a pneumothorax, and a 27-yr-old female sporadic case after a first pneumothorax event and striking parenchymal lung changes on a high-resolution CT (HRCT). Based on the association of lung cysts and pneumothoraces with BHDS, affected family members from two generations and one sporadic case with similar cystic lung structures have been screened for mutations in the *FLCN* gene. Two novel disease-associated DNA sequence alterations were detected.

METHODS

Patient recruitment and examination

All participants gave written informed consent for molecular and clinical testing. Skin changes were excluded by physical examination and kidney manifestations by abdominal ultrasound.

Lung function

Spirometry, whole body plethysmography and measurement of carbon monoxide diffusing capacity ($D_{L,CO}$) were performed (6200 Autobox SensorMedics, Yorba Linda, CA, USA) according to standard criteria [14]. Reference values were in accordance to the European Community for Steel and Coal [15].

CT

Thin-section CT of the chest was performed with a 64-slice CT scanner (Somatom Sensation 64; Siemens Medical Solutions, Forchheim, Germany). Patients were examined in the supine position. Inspiratory scans were obtained during suspended deep inspiration from the apices of the lung to the costophrenic angles. Examination parameters were 120 kV and 150 mAs, using a 512×512 matrix. Images with a slice thickness of 1 mm and an increment of 0.8 mm were reconstructed with a high spatial frequency algorithm and analysed at window settings appropriate for viewing lung parenchyma (window centre -600 HU; window width 1,500 HU). No intravenous contrast material was administered.

Mutation analysis

Genomic DNA was isolated from circulating leukocytes using the chemagic Magnetic Separation Module I (Chemagen Biopolymer-Technology AG, Baesweiler, Germany).

All 11 coding exons and flanking intronic sequences of the *FLCN* gene were amplified and sequenced. PCR amplifications, PCR product purification and cycle sequencing were performed under routine conditions (details are available upon request). The detected sequence variants were verified by repeated sequencing on newly amplified PCR products. The control panel included >210 alleles from unrelated unaffected individuals of the Swiss population.

FLCN transcript analyses

Total RNA was isolated using the PAXgene Blood RNA kit (Qiagen, Hombrechtikon, Switzerland) from venous blood collected into PAXgene Blood RNA Tubes (Becton Dickinson, Basel, Switzerland) according to the manufacturers' instructions. The quality of the purified RNA (as determined by RNA integrity number (RIN)) was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). RNA samples with RIN >5 were used as template for cDNA synthesis, which was carried out with a commercially available kit for reverse transcription using 4 μg total RNA and random primers (Superscript III kit; Invitrogen, Basel, Switzerland).

The amount of *FLCN* transcripts was determined by quantitative real-time RT-PCR performed on an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Rotkreuz, Switzerland) using primers specific for *FLCN* and the endogenous reference *POLR2A* as well as TaqMan probe (Applied Biosystems) or SybrGreen I dye (Applied Biosystems) as a reporter for *FLCN* and *POLR2A*, respectively. Amplicons were run as triplicates and standard curves were prepared for both

FLCN and *POLR2A*. After normalisation to *POLR2A*, the *FLCN* transcript expression was calculated relative to that of a calibrator sample of normal, healthy family member III.1. Repeated observations of relative expressions were analysed by descriptive statistics. For the arithmetic mean, upper and lower confidence limits were calculated using critical values of paired t-test distribution [16].

The ratio of allele-specific transcripts was quantified basically according to a procedure outlined by QIU *et al.* [17] by sequencing of mutation-harboring RT-PCR products on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) using primers specific to exons 6 and 8 (details on reaction conditions are available upon request).

RESULTS

History and clinical features

None of the individuals analysed in the present study had α-antitrypsin deficiency, a connective tissue disorder such as Marfan or Ehlers-Danlos syndrome, nor skin or kidney manifestations of BHDS.

The index patient of the Swiss family (III.3) was a 56-yr-old female (fig. 1) who consulted her family doctor after an episode of shortness of breath during her preceding holidays. Her history is remarkable for a first pneumothorax after giving birth to her oldest child (IV.3), and a second event three weeks before the birth of her second child (IV.4). A HRCT scan of the thorax showed bilateral cystic lung lesions preferentially in both lower lobes within otherwise radiologically normal lung parenchyma (fig. 2a). Her lung function was normal.

The patient's family was of Swiss descent. Deceased family members, *i.e.* her father (II.1), an uncle (II.3) and her grandfather (I.1), were also known to have experienced at least one pneumothorax episode. The patient's brother (III.5) and her
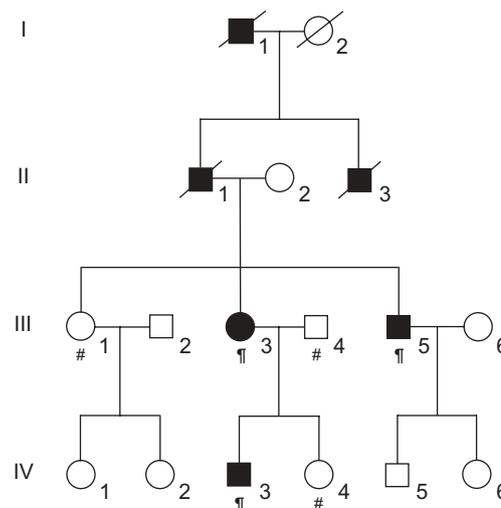
FIGURE 1. Pedigree of a Swiss family with spontaneous pneumothorax. Circles indicate females, squares indicate males, open symbols indicate unaffected individuals and filled symbols indicate affected individuals; symbols crossed through with a line indicate deceased individuals. Generations and individuals are identified by roman and arabic numerals, respectively. The determined genotype is given below each symbol representing investigated individuals. The index patient is III.3. #: [=]+[=]; ¶: [c.779G>A]+[=]. [=]: wildtype.

son (IV.3), both with a typical history for at least one spontaneous pneumothorax event, as well as her healthy sister (III.1) and her daughter (IV.4), who denied previous episodes of chest pain and/or shortness of breath, were available for testing. Except for the nonconsanguineous husband (III.4) of the index patient, all participant were nonsmokers. The lung function, *i.e.* dynamic and static lung volumes, as well as $D_{L,CO}$ was normal in the index patient's sister and her children and revealed mild obstruction to airflow in her brother, who was known to suffer from bronchial asthma. HRCT revealed the same, but less striking, alterations, consisting of small cysts within otherwise unremarkable lung parenchyma, but was completely normal in her son, who had a pneumothorax event at the age of 22 yrs.

The family of the sporadic female case was unknown, since the 27-yr-old nonsmoking female was adopted as a child from Brazil and does not know any consanguineous family members. She experienced pain in her right chest 1 week before admission, followed by shortness of breath, and a right-sided pneumothorax was diagnosed. Since an air leak persisted for 3 days after the insertion of a chest tube, video-assisted thoracoscopy was indicated. Blebs were seen at the pleural surface and a wedge resection of the right upper lobe, a pleural abrasion and talc poudrage for pleurodesis was performed. Histology revealed small blebs within normal lung tissue but without features of lymphangioleiomyomatosis and Langerhans' cell histiocytosis and negative staining for HBM-45 and S-100, respectively. The HRCT of the patient showed small cysts besides normal lung structure (fig. 2b), strikingly similar to the index patient of the large Swiss family.

Mutation analysis of the FLCN gene
Direct sequencing of all 11 coding exons of *FLCN* (exons 4–14) amplified from genomic DNA of three affected family members revealed a novel heterozygous nonsense mutation in exon 7 (c.779G>A, p.W260X; fig. 3c and e). It leads to a premature termination codon at position 260 instead of 581 in the open reading frame of *FLCN* isoform 1. The mutation c.779G>A was present in all three affected family members, while 346 control alleles did not carry this sequence variant. The three affected patients (III.3, III.5 and IV.3) all had at least one spontaneous pneumothorax during their lifetime. In addition, III.3 and III.5, but not IV.3, had abnormal HRCT (see above).

In the sporadic female case, a novel heterozygous missense mutation (fig. 3b and d) was found. The transition c.394G>A in exon 5 leads to a single glutamic acid to lysine substitution at codon position 132 (p.E132K). This substitution affects an evolutionary highly conserved amino acid, indicating an important role of the glutamic acid at position 132 for the correct function of the FLCN protein [10]. In addition, it did not appear in 356 control alleles. According to COLLINS and SCHWARTZ [18], a sequence alteration shows a high probability of being pathogenic if it is not found in >210 control alleles.

The high conservation and the absence of the p.E132K amino acid substitution in the unaffected population suggest it to be disease-causing mutation.

Transcript analyses of FLCN
Premature termination codons are known to cause nonsense-mediated decay (NMD), a mechanism of mRNA surveillance that prevents the expression of truncated proteins. This is achieved by a selective degradation of the respective mRNA molecules. In order to quantify *FLCN* mRNA levels in affected family members carrying the premature termination codon real-time RT-PCR analyses were preformed. RNA extracted from peripheral blood of patient III.5 carrying the heterozygous c.779G>A (p.W260X) mutation revealed a significantly reduced amount of *FLCN* transcripts (mean±SD 43±11%; p=0.05) compared with a healthy family member (III.1; 100±12%; p=0.05; fig. 3f). Semi-quantitative sequencing of RT-PCR products from patient III.5 was also performed; a highly reduced amount of mutated transcripts (A allele) was detected in comparison with wild-type transcripts (G allele at position c.779; data not shown).

DISCUSSION
After the first published observation of an increased frequency of spontaneous pneumothorax in patients with BHDS, other
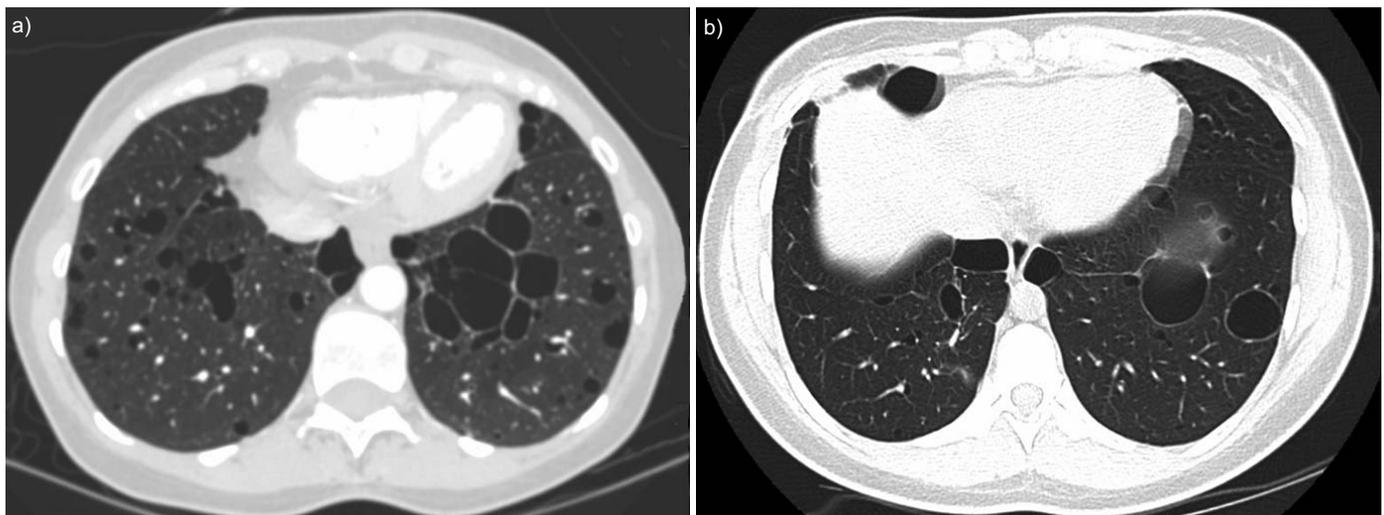
FIGURE 2. a) Chest high-resolution computed tomography of patient III.3. b) Chest high-resolution computed tomography of the sporadic case.
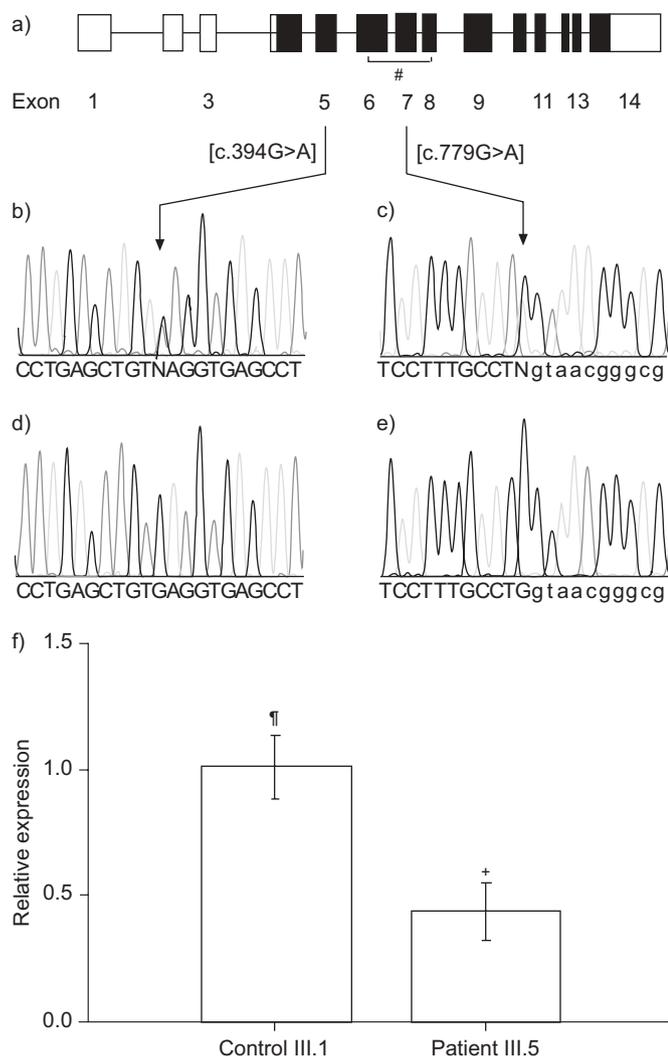
FIGURE 3. a) Exon structure of the folliculin (*FLCN*) gene. Boxed symbols indicate exons, lines indicate introns, filled boxes indicate coding exons, unfilled boxes the 5' and 3' untranslated region. #: position of TaqMan probe used for transcript measurement. Electropherograms showing the two novel *FLCN* mutations in the sporadic case (b) and patient III.3 (c) compared with a control (III.1; d and e). Arrows indicate the site of mutation, lowercase letters indicate intronic sequences, and uppercase letters indicate exonic sequences. f) Relative expression (with confidence intervals) of *FLCN* transcript in patient III.5 compared with unaffected family member III.1 (p=0.05).

individuals with a family history of spontaneous pneumothorax but without clinical features of BHDS have been described. Some of them have shown a mutation in *FLCN*, with lung cysts as the morphological basis for pneumothorax events [12]. The present findings confirm that, in individuals with a family spontaneous pneumothorax history or in persons with a spontaneous pneumothorax and multiple lung cysts, mutations of *FLCN* may be found even in the absence of the typical dermatological findings for BHDS, *i.e.* fibrofolliculomas of the skin.

There are several considerations about the function of FLCN protein in the cell, *e.g.* tumour suppressor activity by involvement in mTOR (mammalian target of rapamycin) signalling has been suggested [19]. Expression studies have revealed that the

folliculin mRNA is widely, but not universally, expressed in human organs and tissues including skin, lung and kidney [20]. In the lung, *FLCN* is transcribed in type-1 pneumocytes and stroma cells, including fibroblasts and macrophages. An imbalance may either induce an inflammatory response or alter matrix degradation and remodelling.

A variety of mutations in all 11 coding exons of *FLCN* have been detected since the first description of the clinical manifestations of mutations in this gene. The most frequent comprise frameshift or nonsense mutations that are predicted to introduce a premature termination codon. However, missense and splice site mutations were also associated with spontaneous pneumothorax [10]. No clear-cut correlation between the type of *FLCN* mutation and the disease phenotype, *i.e.* BHDS or spontaneous pneumothorax without BHDS features, has been found so far. Recently, an extensive investigation of lung cysts and pneumothorax comprising 198 patients in 89 families with BHDS was published. In that study, *FLCN* mutations in exons 9 and 12 were associated with a higher number of cysts, larger cyst diameters and a higher incidence of pneumothorax events [10].

The nonsense mutation in exon 7 (c.779G>A, p.W260X) may lead to NMD [21]. In order to investigate the effect of the nonsense mutation on RNA expression, the amounts of *FLCN* transcript in patient III.5 were compared with an unaffected family member (III.1) of comparable age. Indeed, this analysis revealed a 60% reduction of the transcript level in the patients' RNA, probably due to NMD. The missense mutation c.394G>A (p.E132K) in exon 5 of *FLCN* affects an amino acid residue that is highly conserved across species. Future studies will show whether or not this mutation leads to a reduced amount of *FLCN* transcripts, as reported for missense mutations in other genes [22].

The present findings lead to the conclusion that the pathogenic defect of spontaneous pneumothorax is, at least for the nonsense mutation, due to the lower amount of normal *FLCN* transcript compared with that in unaffected persons. Determination of whether these observations also apply to lung tissues requires the investigation of affected human lung tissues. It would be also of interest if other *FLCN* mutations associated with spontaneous pneumothorax in general leads to an *FLCN* transcript reduction and if this is different in patients with BHDS and *FLCN* mutations.

In patients with spontaneous pneumothorax, CT of the lung shows either a normal lung structure or few blebs in the apical region of the lung. The lung cysts, which may be found in patients with mutations in *FLCN* with or without features of BHDS, are unique and distinct from the cystic lesions found in pulmonary emphysema or in Langerhans' cell histiocytosis, lung diseases where the cystic lesions are accompanied by other radiomorphological alterations typical for the underlying disease. The cysts resemble punched holes in an otherwise normally structured lung and are not preferentially located in the upper lobes of the lung. As recently described in 48 patients with BHDS with a history of pneumothorax, the cysts may vary in size from a few millimetres to several centimetres and number from a few up to >100 [10]. Lymphangioleiomyomatosis, a disease almost exclusively affecting women in the third and fourth decade of life, may be more difficult to distinguish radiologically from

BHDS, since in this disease the cysts also may vary in size and are randomly distributed throughout the lungs with no noticeable changes in the intervening lung parenchyma.

In a retrospective study in Mayo Clinic (Rochester, MN, USA), including five patients during an 8-yr period (1998–2005), pulmonary function results were available in four patients and reported to be normal in the one single patient who never smoked [23]. The present study is the first in which lung function measurements were performed systematically in nonsmoking individuals with an folliculin gene defect. The functional data are consistent with the radiomorphological findings and indicate that, apart from lung cysts, the pulmonary parenchyma in the investigated patients seem not to be defective, which is in contrast to other cystic lung diseases, such as lymphangioleiomyomatosis, Langerhans' cell histiocytosis, and pulmonary emphysema.

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