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Novel mutations in the folliculin (*FLCN*) gene associated with spontaneous pneumothorax

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ABSTRACT

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

Aim: To investigate the presence and type of mutation in a Swiss pedigree and in a sporadic case.

Methods: Clinical examination, lung function tests and HRCT. 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.

Results: We identified two novel mutations in FLCN. Three investigated family members with a history of at least one SP 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 to 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 the CT showed similar bilateral cysts as observed in the two members of the unrelated Swiss family.

Conclusions: Mutations in FLCN are associated with cystic lung lesions in an otherwise morphological normal lung and predispose to SP.

Abbreviations

SP: Spontaneous pneumothorax; BHDS: Birt-Hogg-Dubé syndrome; FLCN: folliculin; PCR: polymerase chain reaction; HRCT: high-resolution computed tomography; UTR: untranslated region; NMD: nonsense mediated mRNA decay.

A spontaneous pneumothorax (SP) 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 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 inheritance have been suggested [5, 6, 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 gene (*FLCN*) 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 oncologic manifestations of BHDS [12, 13].

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

METHODS

Patient Recruitment & Examination

All participants gave written informed consent for molecular und 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 (DLco) 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].

Computed tomography

Thin-section computed tomography (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 analyzed at window settings appropriate for viewing lung parenchyma (window center –600 HU; window width, 1500 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 eleven 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 (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 (RT) using 4µg 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 (*FLCN*) or SybrGreen I dye (*POLR2A*) as a reporter. Amplicons were run as triplicates and standard curves were prepared for both *FLCN* and *POLR2A*. After normalization 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 analyzed by descriptive statistics. For the arithmetic

mean, upper and lower confidence limits were calculated using critical values of Student`s t-distribution [16].

The ratio of allele-specific transcripts was quantified basically according to a procedure outlined by Qiu *et al*. 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) [17].

RESULTS

History and clinical features

None of the individuals analyzed in this 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) is a 56-year-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 shows bilateral cystic lung lesions preferentially in both lower lobes within otherwise radiological normal lung parenchyma (fig 2a). Her lung function is normal.

The patient's family is of Swiss descent. Deceased family members as her father (II.1), an uncle (II.3), and her grandfather (I.1) are also known to have experienced at least one pneumothorax episode. The patient's brother (III.5) und her son (IV.3), both with a typical history for at least one SP 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 the non consanguineous husband (III.4) of the index patient all participant are non-smokers. The lung function i.e. dynamic and static lung volumes as well as diffusing capacity for carbon monoxide was normal in the index patients sister and her children and revealed mild obstruction to airflow in her brother, who is 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 years.

The family of the sporadic female case is unknown, since the 27-year-old nonsmoking woman has been adopted as a child from Brazil and does not know any consanguineous family members. One week before admission she experienced pain in her right chest followed by shortness of breath and a right sided pneumothorax was diagnosed. Since an air leak persisted for three 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 talk 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 this patient shows 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 eleven 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 3B). 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, IV.3) had all at least one SP during their lifetime. In addition, III.3 and III.5 but not IV.3 had an abnormal HRCT (see above).

In the sporadic female case we found a novel heterozygous missense mutation (fig 3B). The transition c.394G>A in exon 5 leads to a single glutamic acid to a 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 a sequence alteration shows a high probability to be pathogenic if it is not found in more than 210 control alleles [18].

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

Transcript Analyses of *FLCN*

Premature termination codons are known to cause nonsense mediated decay (NMD), a mechanism of mRNA surveillance to prevent 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 we performed real-time RT-PCR analyses. 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 (43±11%, P=0.05) compared to a healthy family member (III.1) (100±12%; P=0.05) (fig 3C). We also performed semi-quantitative sequencing of RT-PCR products from patient III.5 and detected a highly reduced amount of mutated transcripts (A allele) in comparison to wild-type transcripts (G allele at position c.779, data not shown).

DISCUSSION

After the first published observation of an increased frequency of SP in patients with BHDS other individuals with a family history of SP but without clinical features of BHDS have been described. Some of them showed a mutation in the folliculin gene, *FLCN*, with lung cysts as the morphological basis for pneumothorax events [12]. Our findings confirm that in individuals with a family SP history or in persons with a SP and multiple lung cysts, mutations of *FLCN* may be found even in the absence of the typical dermatologic 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 signalling has been suggested [19]. Expression studies 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 eleven coding exons of the folliculin gene 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, also missense and splice site mutations were associated with SP [10]. No clear cut correlation between the type of folliculin mutation and the disease phenotype, i.e. BHDS or SP without BHDS features has been found so far. Recently, an extensive investigation on lung cysts and pneumothorax comprising 198 patients

in 89 families with BHDS was published. In this 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 nonsense mediated mRNA decay (NMD) [21]. To investigate the effect of the nonsense mutation on RNA expression, we compared the amounts of *FLCN* transcript in patient III.5 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, which is highly conserved across species. Future studies will show whether or not this mutations leads to a reduced amount of *FLCN* transcripts as reported for missense mutations in other genes [22].

Our findings lead to the conclusion that the pathogenic defect of SP is at least for the nonsense mutation due to the lower amount of normal *FLCN* transcript compared to unaffected persons. Discovery whether our observation also apply to lung tissues require the investigation of affected human lung tissues. It would be also of interest if other *FLCN* mutations associated with SP in general leads to an *FLCN* transcript reduction and if this is different in patients with BHDS and *FLCN* mutations.

In patients with SP computed tomography 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 from a few up to over 100 [10]. Lymphangioleiomyomatosis, a disease almost exclusively affecting women in the third and fourth decade of her life, may be more difficult to distinguish radiologically from the BHD syndrome, since also in this disease the cysts may vary in size and are randomly distributed throughout the lungs with no noticeable changes in the intervening lung parenchyma.

In a retrospective study of the Mayo Clinic, including five patients during an 8-year period (1998-2005), pulmonary function results were available in four patients and reported to be normal in the one single patient, who never has smoked [23]. Our study is the first, where lung function measurements were performed systematically in non-smoking individuals with a folliculin gene defect. The functional data are consistent with the radiomorphologic findings and indicate that apart from lung cysts the pulmonary parenchyma in the investigated patients seems not defective, which is in contrast to other cystic lung diseases such as lymphangioleio-myomatosis, Langerhans cell histiocytosis, and pulmonary emphysema.

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COMPETING INTERESTS

None.

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LEGENDS TO FIGURES

Figure 1 Pedigree of a Swiss family with SP. Circles indicate females, squares indicate males, open symbols indicate unaffected individuals, filled symbols indicate affected individuals, and lines across symbols 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.

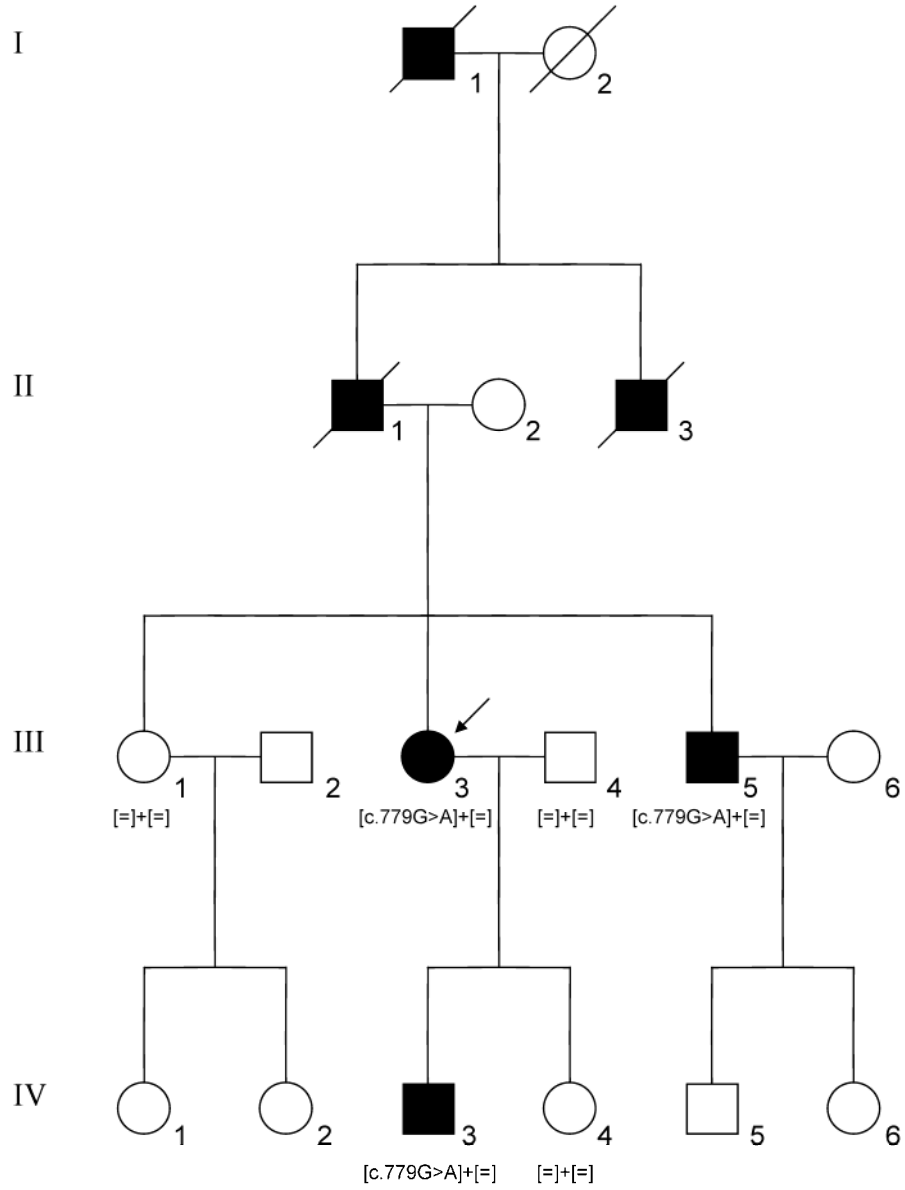

I
1
2
II
1
2
3
III
1
[=]+[=]
2
3
[c.779G>A]+[=]
4
[=]+[=]
5
[c.779G>A]+[=]
6
IV
1
2
3
[c.779G>A]+[=]
4
[=]+[=]
5
6

Figure 2a Chest high-resolution computed tomography of patient III.3.

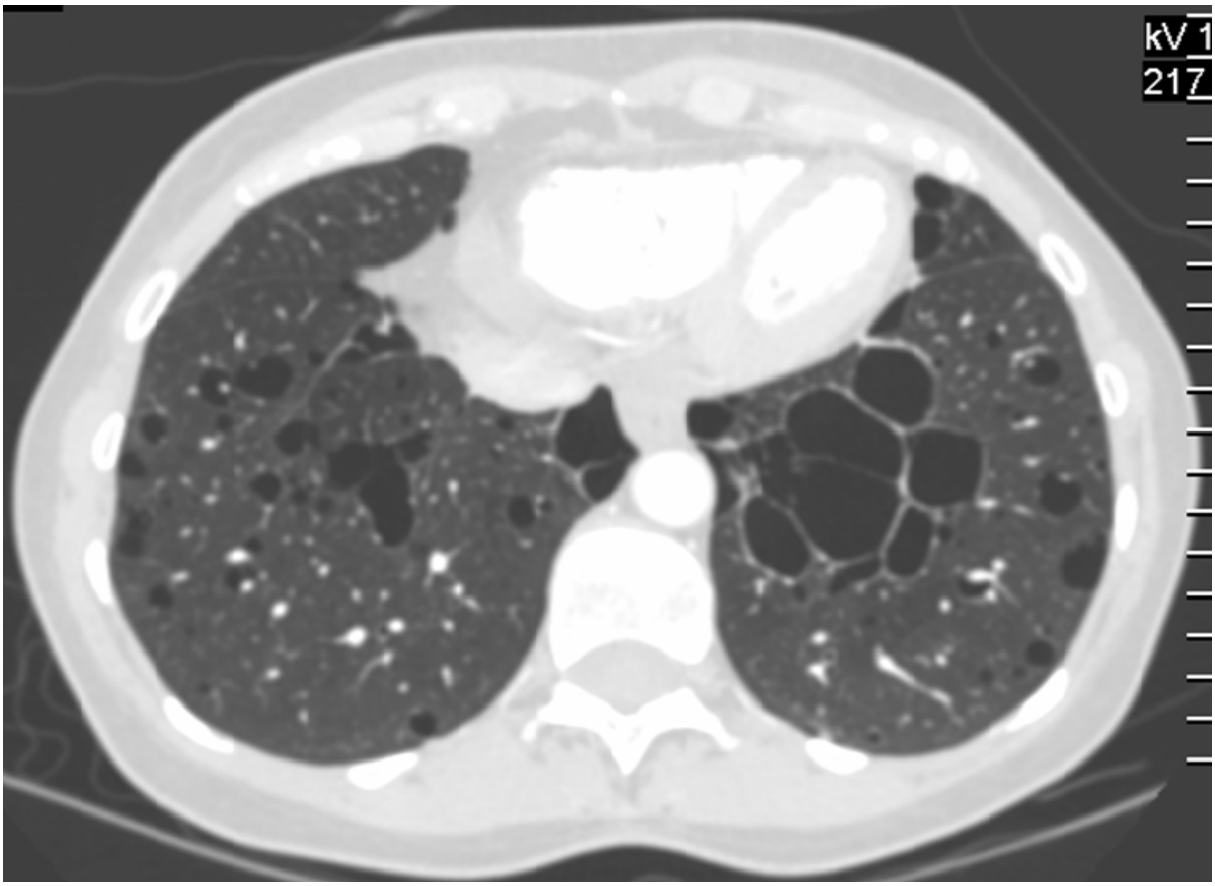


Figure 2b Chest high-resolution computed tomography of the sporadic case.

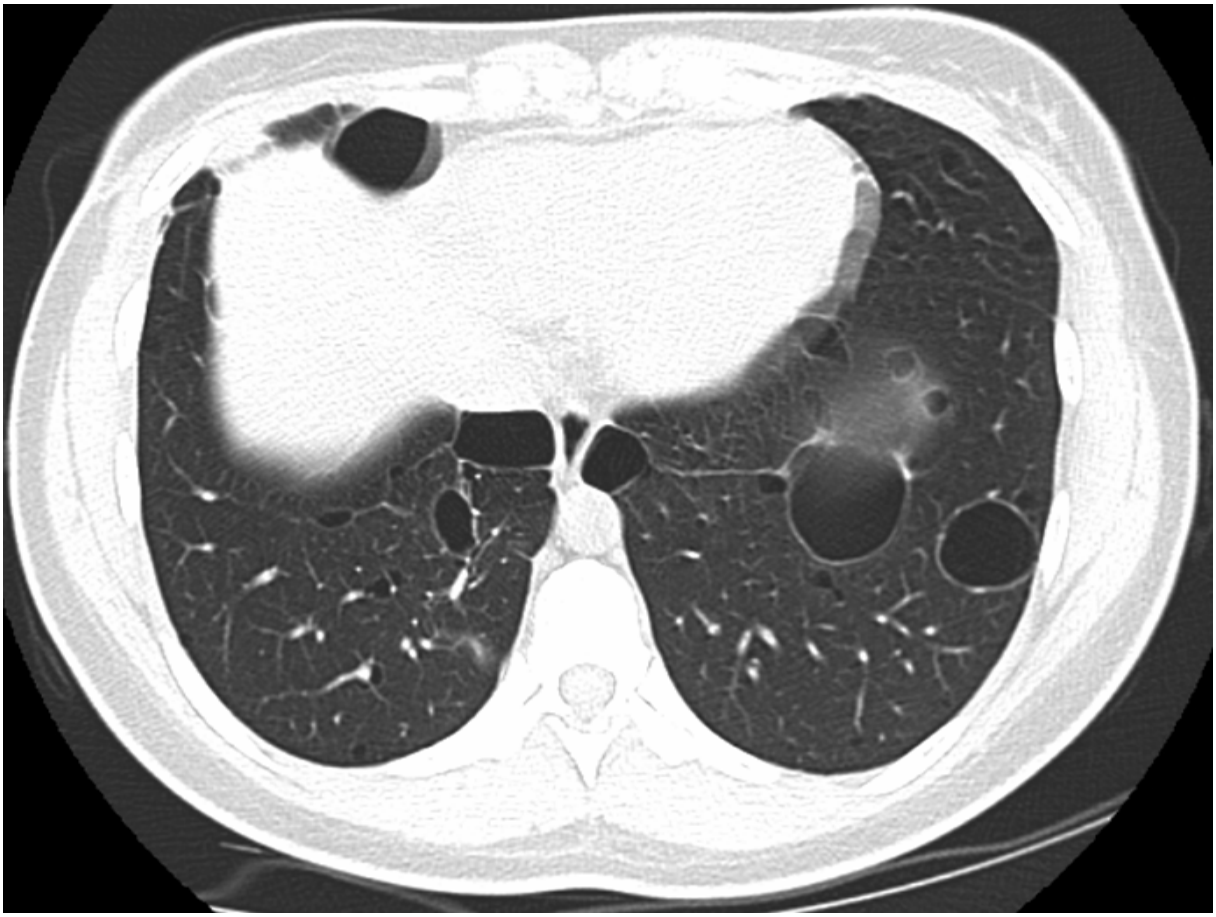
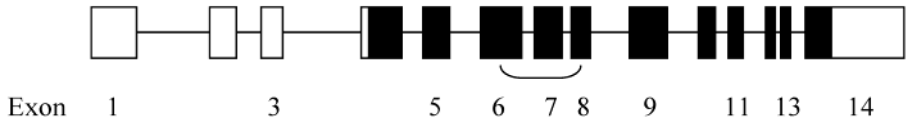
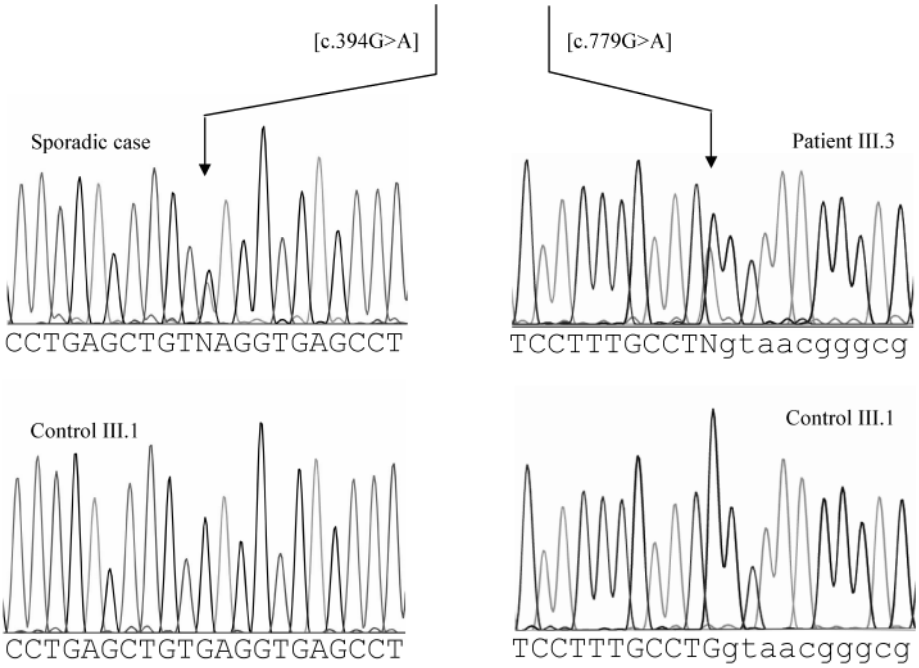


Figure 3 *A*, Exon structure of *FLCN*. Boxed symbols indicate exons, lines indicate introns, filled boxes indicate coding exons, unfilled boxes the 5' and 3' UTR, waved caret indicates the position of TaqMan probe used for transcript measurement. *B*, Electropherograms showing the two novel *FLCN* mutations compared with a control. Arrows indicate the site of mutation, lowercase letters indicate intronic sequences, and uppercase letters indicate exonic sequences. *C*, Relative Expression of *FLCN* transcript in patient III.5 compared to unaffected family member III.1. Vertical lines indicate confident intervals (P=0.05).

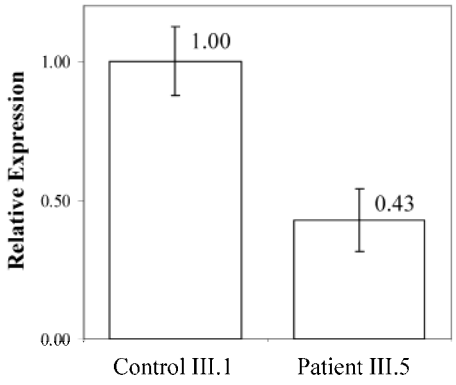
A


Exon 1 3 5 6 7 8 9 11 13 14

B


[c.394G>A]
[c.779G>A]
Sporadic case
Patient III.3
CCTGAGCTGTNAGGTGAGCCT
TCCTTTGCCTNgtaacgggcg
Control III.1
Control III.1
CCTGAGCTGTGAGGTGAGCCT
TCCTTTGCCTGgtaacgggcg

C


Relative Expression
1.00
0.50
0.00
1.00
0.43
Control III.1
Patient III.5