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Short Report

Mutation analysis of the *FLCN* gene in Chinese patients with sporadic and familial isolated primary spontaneous pneumothorax

Ren H-Z, Zhu C-C, Yang C, Chen S-L, Xie J, Hou Y-Y, Xu Z-F, Wang D-J, Mu D-K, Ma D-H, Wang Y, Ye M-H, Ye Z-R, Chen B-F, Wang C-G, Lin J, Qiao D, Yi L. Mutation analysis of the *FLCN* gene in Chinese patients with sporadic and familial isolated primary spontaneous pneumothorax.
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Primary spontaneous pneumothorax (PSP) is a common manifestation of Birt–Hogg–Dubé syndrome caused by folliculin gene (*FLCN*) mutation, which is also found in isolated familial PSP cases. A complete genetic analysis of *FLCN* was performed in 102 unrelated Chinese patients with isolated PSP and 21 of their family members. Three novel mutations (c.924_926del, c.1611_1631del and c.1740C>T) and a previously reported mutation (c.1733insC) were identified in five familial and five sporadic PSP patients. Of the 21 family members of patients with PSP including 3 previous considered as sporadic, 4 (19%) had history of at least one episode of PSP and 9 (43%) were *FLCN* mutant carriers without PSP. Seven of the nine (78%) mutant carriers had pulmonary cysts detected by high-resolution computed tomography (HRCT). Although c.924_926del and c.1611_1631del were found in eight patients from the same geographic district, haplotype analysis demonstrated that they did not share the same affected haplotype, thus excluding common ancestry. This study first demonstrates that *FLCN* mutation contributes to not only familial but also 'apparently sporadic' patients with isolated PSP. It suggests that mutation analysis and HRCT scan may be recommended for first-degree family members of PSP patients with *FLCN* mutations, irrespective of their family history status of PSP.

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Key words: *FLCN* gene – mutation – primary spontaneous pneumothorax

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Primary spontaneous pneumothorax (PSP; MIM 173600) results from the rupture of peripheral pulmonary cysts and contributes to secondary collapse of the lung and hypoxia. It has been noted that most PSP cases are sporadic and only 11.5% patients have a positive family history (1). The genetic etiology in most cases remains unclear, although PSP is a complication of certain genetic

disorders including Marfan syndrome, cystic fibrosis, homocystinuria, Ehlers–Danlos syndrome, α1-Antitrypsin deficiency and Birt–Hogg–Dubé syndrome (BHDS) (2).

Folliculin gene (*FLCN*; MIM 607273) is located on chromosome 17p11.2 and encodes a deduced 579-amino acid protein designated folliculin. Mutations in this gene are responsible for BHDS, which is a rare inherited genodermatosis characterized by hair follicle hamartomas, spontaneous pneumothorax and kidney tumors (3–5). In a recent study, 24% (48/198) of patients with BHDS had a history of pneumothorax and have a significant association between the diameter, number and location of lung cysts and spontaneous pneumothorax (6). Isolated PSP patients with *FLCN* mutations have been reported in a Finnish family (7), two American PSP families (8) and five Japanese PSP families (9). Interestingly, except for PSP, no other features of BHDS were detected in any cases in these reports. Although the function of the FLCN protein is currently unknown, the study of *FLCN* mRNA has revealed that in the lung tissue it is expressed strongly in stromal cells (macrophage, fibroblasts and lymphocytes) and moderately in the type 1 pneumocytes (10), suggesting that *FLCN* mutation may either induce inflammatory response or alter matrix degradation and remodeling in lung tissue.

Based on the evidence mentioned above, *FLCN* mutation is considered to be molecular basis for at least some PSP cases. To investigate the contribution of *FLCN* mutations to both familial and sporadic PSP, we conducted a *FLCN* gene mutational analysis in a group of 102 PSP cases from the Chinese Han population.

Materials and methods

Subjects

From January 2003 to January 2007, blood samples were collected from 102 unrelated patients (94 males and 8 females, aged from 15 to 67 years) who were admitted with PSP episodes to two tertiary hospitals, Taizhou Hospital in Linhai of Zhejiang Province and Drum Tower Hospital in Nanjing of Jiangsu Province. Ten patients had a familial history of PSP and 92 were sporadic. After a mutation survey of these patients was completed, genetic analysis was extended to 21 family members of 7 mutant patients (family members of the other 3 were unavailable). Ten mutant patients and 21 of their family members underwent thorough skin examination, high-resolution computed tomography (HRCT) and abdominal and kidney ultrasonography.

As a control group, 120 unrelated healthy individuals were enrolled at the Center of Physical Examination of Drum Tower Hospital. The control group had never had any histories, signs or symptoms of lung diseases and tumors by medical questionnaire, physical examination, thoracic X-ray scan and abdominal ultrasonography.

The study was approved by the Ethical Committee of Nanjing University Medical School and Taizhou Hospital. Informed consent was obtained from controls, patients and their family members.

DNA isolation and amplification of *FLCN* fragments

Genomic DNA was prepared from peripheral blood leukocytes by using Chelex-100 method (11) or the DNA IQ system (Promega, Madison, WI) according to the manufacturer's instructions.

The complete coding sequence of the *FLCN* gene was amplified. Exons 5, 6, 7, 10 and 11 were amplified using the primers described elsewhere (4). The primers of exons 4, 8, 9, 12, 13 and 14 were redesigned for optimal size of single-strand conformation polymorphism (SSCP) sensitivity (12) based on genomic sequences (GenBank accession number AC055811) by using the online software PRIMER3 (13). The redesigned primer sequences are available upon request. The amplification reaction mixture (25 μl) was subjected to denaturation at 95°C for 2 min followed by 30 cycles at 94°C for 1 min, annealing temperature 60 or 64°C for 1 min, 72°C for 1 min and by a final extension at 72°C for 15 min.

Detection, confirmation and analysis of mutations

To identify the mutation of the studied exons, SSCP analysis was carried out on the samples. In brief, denatured products were resolved by electrophoresis on 8% non-denaturing polyacrylamide gels for 16–18 h at 8–10°C and were visualized by silver staining. Once an abnormal fragment was revealed from the SSCP, it was purified and sequenced double stranded on an ABI 3130 automated sequencer using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). Sequence alterations were verified when possible by 6% denaturing polyacrylamide gel, which was performed as described elsewhere (14). Mutations are described according to the recommended nomenclature at http://www.hgvs.org/mutnomen (15). Nucleotide numbers are derived from GenBank accession number AF517523 assuming that the nucleotide 456 is the A of the first ATG translation initiation codon.

Haplotype analysis

Haplotype analysis was performed to determine if mutations had a common founder. Genomic DNA of patients with mutations and their family members was genotyped with three microsatellite markers (17_5_1, D17S740 and D17S2196) and four single nucleotide polymorphisms (rs17794784, rs1736219, rs1708620 and rs1736210), which span about 0.78 Mb within or surrounding the *FLCN* gene.

Results

In this study, 10 PSP patients and 13 of their family members were demonstrated to have a heterozygous *FLCN* mutation (Table 1), and no mutation was identified in the controls. Of the 10 patients with mutations, half have family PSP histories. Among the 23 individuals with mutations, there were 14 with PSP and 9 (7 with cysts and 2 without cysts) without PSP. Seven of these individuals had a smoking history. A family pedigree illustrates the different findings of mutation, PSP and cysts in F74's family (Fig. 1a). No clinical evidence of hair follicle hamartomas and kidney tumors was discovered in 10 PSP patients and 21 of their family members.

A total of three novel heterozygous *FLCN* mutations (c.924_926del, c.1611_1631del and c.1740C>T) and one previously known heterozygous mutation (c.1733insC) were identified in this study (Table 1). The first novel mutation, c.924_926del, consists of a 3-bp in-frame deletion in exon 6 of the *FLCN* gene (Fig. 1b,c). This change generates a phenylalanine (TTC) deletion at codon 157. The second is a frameshift mutation resulting from the 20 bp deletion in exon 10 (Fig. 2). The mutation produces a stop codon (TGA) 187 nt downstream. The third is a missense mutation

Table 1. Summary of clinical and mutation data of 10 PSP patients and their family members

No.	Patients and family members	Sex	Age[a]	Family history	No. of episodes	Smoking history	Location, diameter, number of pulmonary cysts	Exon	Mutation
1	F23	M	30 (30)	None	1	15 pack-years	Surface of the left lung, 1–5 cm, 10	6	c.924_926del
	F23-I.2, mother	F	52		None	Never	Right apical, 1–3 cm, 3	6	c.924_926del
2	F47	F	43 (39)	Yes	2	Never	Right lingula and apical, 1–3 cm, 10	6	c.924_926del
3	F74	M	51 (51)	Yes	1	17 pack-years	Both lungs, 1–5 cm, >10	6	c.924_926del
	F74-I.2, mother	F	72		None	Never	Both lungs, 2–3 cm, 5	6	c.924_926del
	F74-II.2, brother	M	34 (28)		1	Never	Left upper lung, 2 cm, 1	6	c.924_926del
	F74-II.3, sister	F	49		None	Never	Left upper lung, 2 cm, 1	6	c.924_926del
	F74-III.1, daughter	F	26		None	Never	None	6	c.924_926del
	F74-III.2, daughter	F	24		None	Never	Left upper lung, 1 cm, 2	6	c.924_926del
4	F89	F	27 (26)	None	2	Never	Surface of both lungs, 1–3 cm, >10	6	c.924_926del
	F89-I.1, father	M	52		None	30 pack-years	None	6	c.924_926del
	F89-II.1, sister	F	30		None	Never	Surface of both lungs, 1–3 cm, >10	6	c.924_926del
5	F14	M	28 (28)	None	1	Never	Left apical, 2–3 cm, 3	10	c.1611_1631del
6	F31	M	41 (39)	Yes	3	20 pack-years	Both upper lungs, 1–3 cm, >10	10	c.1611_1631del
	F31-II.1, brother	M	56 (26)		2	10 pack-years	Both upper lungs, 1–3 cm, 5	10	c.1611_1631del
7	F93	M	42 (42)	None	1	40 pack-years	Surface of both lungs, 1–5 cm, >10	10	c.1611_1631del
	F93-I.2, mother	F	66		None	Never	Right lingula, 3 cm, 1	10	c.1611_1631del
8	F98	M	28 (28)	Yes	1	Never	Surface of both lungs, 1–5 cm, 10	10	c.1611_1631del
	F98-I.2, mother	F	53		1	Never	Left apical, 3 cm, 3	10	c.1611_1631del
9	F86	M	67 (67)	None	1	Never	Left lower lung, 12 cm, 1	11	c.1740C>T
10	F27	M	68 (67)	Yes	1	Never	Surface of both lungs, 1–3 cm, >10	11	c.1733insC
	F27-2.1, daughter	F	44		None	Never	Left apical, 1–2 cm, 2	11	c.1733insC
	F27-2.2, son	M	42 (40)		1	10 pack-years	Right apical, 3 cm, 1	11	c.1733insC

F, female; M, male.
[a]Age (years) at enrollment in the study is shown. Age at the first PSP episode is indicated in parentheses.

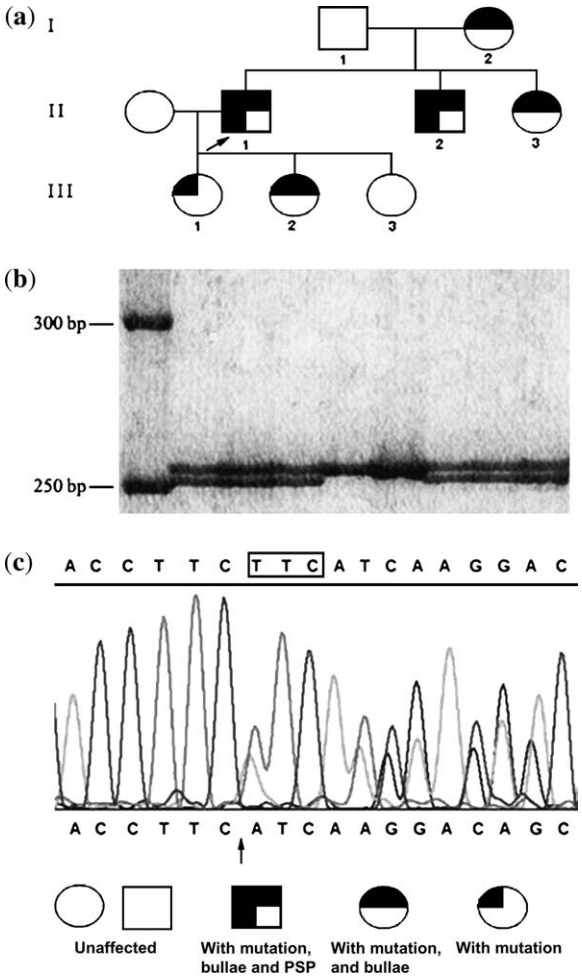

Fig. 1. Mutation analysis of a novel mutation c.924_926del in F74's family. (**a**) The family pedigree. Generation identifier numbers (I–III) are located to the left of each generation and identifier numbers are listed below each family member. Quadrants within pedigree symbols indicate different findings of mutation, cysts and PSP. (**b**) Polymerase chain reaction (PCR) products from the exon 6 amplicon were electrophoresed on a 6% denaturing polyacrylamide gel to separate the 3-bp deletion allele (251 bp) from the wild-type allele (254 bp). Each lane corresponds to the pedigree symbol directly above it. Lane 1, 50 bp DNA Ladder Marker (Takara, Dalian); (**c**) Sequence analysis of a PCR product from the affected F74 (II-1) shows a 3-bp in-frame heterozygous deletion (c.924_926) in exon 6, resulting in unclear multiple chromatograms. The sequence above the waves is wild-type sequence and below the waves is mutant-type sequence. Boxed TTC indicates the 3-bp deletion. An arrow indicates the location of the deletion.

in exon 11, which results in the substitution of histidine by tyrosine at codon 429 (Fig. 3). Mutation c.1733insC is the most commonly reported frameshift in exon 11(5), which creates an early stop codon (TGA) 77 nt downstream in *FLCN* gene.

Mutation c.924_926del was identified in families F23, F74 and F89 and mutation c.1611_1631del in

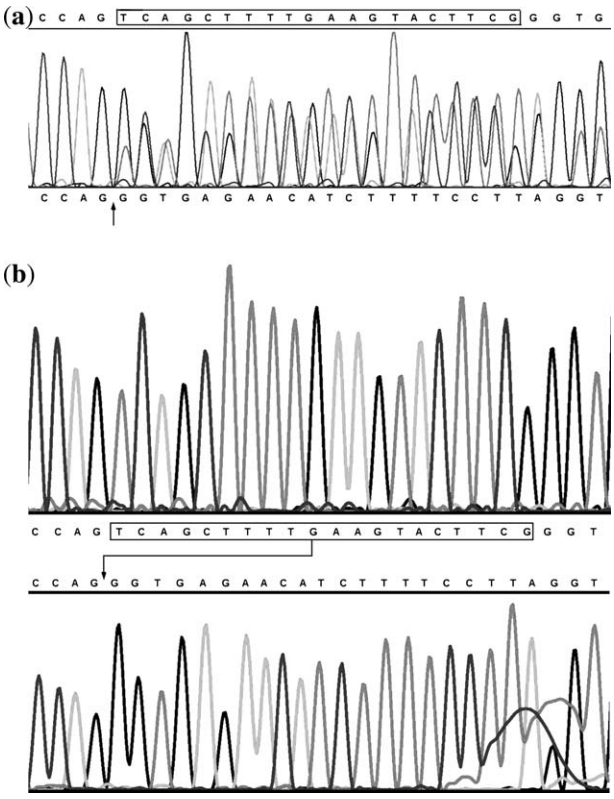

Fig. 2. Sequencing chromatograms of a novel mutation c.1611_1631del in F31. (**a**) Sequencing chromatograms of a polymerase chain reaction (PCR) product from the exon 10 amplicon shows a 20-bp heterozygous frameshift deletion in exon 10, resulting in unclear multiple chromatograms. The sequence above the waves is wild-type sequence and below the waves is mutant-type sequence. Boxed nucleotides indicate the 20-bp deletion. An arrow indicates the location of the deletion. (**b**) The PCR products were electrophoresed on a 3.5% agarose gel to separate the 20-bp deletion allele from the wild-type allele. Then, the two alleles were eluted from the gels, purified and sequencing, respectively. A chromatogram of the wild-type allele is shown in the above and the mutant-type allele in the blow. Boxed nucleotides indicate the 20-bp deletion and an arrow indicate the location of the deletion.

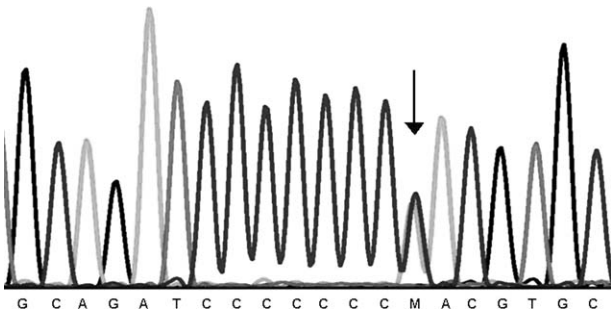

Fig. 3. Sequencing chromatograms of a novel heterozygous mutation c.1740C>T in F86. An arrow indicates position of nucleotide mutation that results in the substitution of histidine by tyrosine at codon 429.

F31, F93 and F98 families. These families came from the same geographic district. Haplotype analysis demonstrated that the three families with c.924_926del mutation and three families with the c.1611_1631del mutation did not share the same affected haplotype, respectively, thus excluding common ancestry (Table 2).

Discussion

PSP is a rare disorder. The incidence is reported to be about 7.4–18/100,000 for men and 1.2–6/100,000 for women (16). PSP has high recurrence rates that range from 30 to 60% in the UK (17) and 13–50% in China (18). Ten of 102 patients in this study had a positive familial history, which is similar to the report by Abolnik et al. (1). The genetic and molecular basis for PSP has not been elucidated. This is the first study at the molecular level about the *FLCN* gene in a group of Chinese patients with PSP. It is noteworthy that novel mutations have been identified in the Chinese population.

PSP is the initial presenting manifestation in BHDS, while the skin and renal findings can develop with age (19–21). In this study, except for PSP or pulmonary cysts, 10 patients and 13 of their family members with mutations did not have any other features of BHDS. Previously, it has been reported that a total of eight families with isolated PSP were affected from *FLCN* mutations (7–9). However, the cases from these cross-sectional studies with lung phenotype without skin or renal manifestations probably are undetected cases of BHDS. They may develop other manifestations with time. Therefore, prospective follow-up clinical studies of this group of patients are needed to better understand how they fit within the spectrum of clinical manifestations of BHDS (22).

We have found four mutations of *FLCN*, including three novel ones (an in-frame triplet deletion, a frameshift deletion and a missense). They can result in an amino acid deletion, a truncated protein or a substitution of an amino acid. The novel mutation c.924_926del consists of an in-frame triplet deletion in exon 6, which is next to the N-terminal of the FLCN. Its potential effect on the conformation or function of FLCN will be investigated further. Most of mutations previously reported (5, 7, 8, 19) were predicted to produce a C-terminal truncated FLCN. Among the mutations leading to truncated FLCN, an insertion (1733insC) and a deletion (1733delC) in a hypermutable C8 tract accounted for 44% cases of BHDS (4). In our study, the novel mutation c.1611_1631del in exon 10 also generates a stop codon (TGA) at the same location (c.1817-c.1819) as the 1733insC and 1733delC, thus producing a premature protein of the same size. A 1733insC mutation previously reported (5) was also identified in a Chinese family, and a novel mutation 1740C>T in a sporadic case is just within C8 tract. Our study supports previous findings of C8 track as a mutational hot spot, with a wide and global distribution (5, 18, 19, 23).

The c.924_926del and c.1611_1631del account for 80% of the mutations we identified. Although the patients with these two mutations came from the same geographic district in China, our result did not demonstrate that they shared a common ancestry. Therefore, it is not clear if these two mutations leading to PSP are new mutational hot spots in the *FLCN* gene. Further studies involving a larger scale and diverse population may be needed to address the issue.

Among the 13 family members with mutations including those of the 3 patients (F23, F89 and F93) previously considered as sporadic, although 9 of them had not developed pneumothorax, 7 had pulmonary cysts. Our findings are consistent with previous studies (7, 21). In the families with BHDS, only 32% of patients with mutations developed pneumothorax, but over 80% have pulmonary

Table 2. The results of genotyping and haplotype analysis for founder effects[a]

	Family with c.924_926del			Family with c.1611_1631del		
Markers	F23	F74	F89	F31	F93	P98
17_5_1	**390**/385	**385**/390	**385**/390	**385**/390	**385**/395	**385**/390
D17S740	**124**/134	**134**/124	**124**/94	**134**/134	**134**/94	**124**/124
rs17794784	**G**/G	**C**/G	**C**/C	**G**/G	**G**/C	**G**/G
rs1736219	**A**/A	**G**/A	**G**/G	**G**/A	**A**/G	**A**/A
rs1708620	**G**/A	**G**/A	**G**/G	**G**/A	**A**/G	**A**/A
rs1736210	**G**/T	**G**/T	**G**/G	**G**/T	**T**/G	**T**/T
D17S2196	**277**/269	**269**/277	**277**/269	**277**/281	**277**/269	**277**/269

[a]*FLCN* is located between rs17794784 and rs1736210. Bold numbers represent the affected haplotype. The naming of the novel marker 17_5_1: firstly, 17 represents chromosome 17, subsequent _5 indicate the number of nucleotides in repetition units and the last part _1 represents serial numbers.

cysts detected by HRCT scans (21). We first revealed that these 'apparently sporadic' patients actually inherited mutations from their mutant parents without PSP. This indicates that *FLCN* mutation could transmit in the family without any clue of PSP family history because of reduced penetrance of the PSP phenotype. Previous researches have identified that smoking is a risk factor for PSP (24). In this study, only 1 of 9 (11%) carriers had a smoking history, while for patients with PSP this figure was as high as 6 of 14 (43%). However, in a large cohort of patients with BHDS, findings of Toro et al. (6) did not support the view that smoking influences the penetrance of PSP phenotype. In their study, 32 of 48 (67%) patients with BHDS who had a history of pneumothorax were non-smokers and 91 of 150 (61%) patients without a pneumothorax were also non-smokers. The inconsistent results between Toro et al.'s study and ours are presumably because of the different study sizes and populations.

This study first demonstrates that *FLCN* mutation contributes to not only familial but also 'apparently sporadic' patients with isolated PSP. It suggests that mutation analysis and HRCT scan may be recommended for first-degree family members of PSP patients with *FLCN* mutations, irrespective of their family history status of PSP.

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