Promoter methylation is not associated with FLCN irregulation in lung cyst lesions of primary spontaneous pneumothorax

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Abstract. Germline mutations in FLCN are responsible for ~10% of patients with primary spontaneous pneumothorax (PSP), characterized by multiple lung cysts in the middle/lower lobes and recurrent pneumothorax. These clinical features are also observed in a substantial portion of patients with sporadic PSP exhibiting no FLCN coding mutations. To assess the potential underlying mechanisms, 71 patients with PSP were selected, including 69 sporadic and 2 familial cases, who bared FLCN mutation-like lung cysts, however, harbored no FLCN protein-altering mutations. Notably, in a significant proportion of the patients, FLCN irregulation was observed at the transcript and protein levels. Genetic analyses of the cis-regulatory region of FLCN were performed by sequencing and multiplex ligation-dependent probe amplification assay. No inheritable DNA defect was detected, with the exception of a heterozygous deletion spanning the FLCN promoter, which was identified in a family with PSP. This mutation caused a reduction in the expression of FLCN in the lung cysts. Pedigree analysis demonstrated that haplinsufficiency of FLCN was pathogenic. To determine whether epigenetic mechanisms may be involved in the irregulation of FLCN, the promoter methylation status was measured in the remainder of the patients. No evidence of FLCN promoter methylation was demonstrated. The present study suggested that FLCN irregulation in lung cysts of PSP is not associated with promoter methylation.

Introduction

Primary spontaneous pneumothorax [PSP; Online Mendelian Inheritance in Man (OMIM)#173600] occurs in patients without clinically apparent underlying lung disease. Of PSP cases, ~10% are caused by germline mutations in the FLCN gene (1,2), which is known to cause Birt-Hogg-Dube syndrome (BHD; OMIM#135150), an autosomal dominant condition characterized by skin fibrofolliculomas, pulmonary cysts/spontaneous pneumothorax and renal cancers (3,4). FLCN mutation may result in isolated PSP with no skin or renal manifestations, presumably due to an incomplete penetration, as has been reported in multiple previous case studies (1,2,5-7).

Lung cysts are the common pathogenic ground of PSP (8,9). Multiple lung cysts in patients with FLCN mutations are often observed randomly and bilaterally distributed in the lung, particularly in its lower portion (10-13). By contrast, apical bullae are often observed in patients with sporadic PSP without FLCN mutations (14-16). Notably, FLCN mutation-like lung cysts are also observed in a significant portion of non-FLCN mutant sporadic PSP cases (6). Whether these cases are in any way associated with FLCN disruption remains to be elucidated.

The FLCN gene (OMIM#607273) encodes an evolutionarily conserved protein, folliculin, with no apparent functional motif currently recognized. The majority of the pathogenic FLCN mutations, identified to date, resulted in premature truncation of the protein (http://www.skingendatabase.com/) (17). FLCN missense mutations and small in-frame deletions were reported to predominantly disrupt protein stability and lead to significant reductions in the expression of FLCN (18). No evidence for a dominant negative effect of FLCN mutants was observed in the transfected cells. Additionally, large intragenic deletions spanning the putative FLCN promoter region have been reported in families with BHD. Luciferase reporter assays demonstrated that a deletion of the putative promoter dramatically reduced the gene expression in vitro (19). In addition, FLCN inactivation caused by promoter methylation has also been detected in types of renal tumor (20,21). This raised the possibility...
that epigenetic regulation of \textit{FLCN} may contribute to the pathology of BHD.

The present study selected 71 patients with PSP, who harbored a \textit{FLCN} mutation-like lung phenotype, however, exhibited no germline and somatic mutations in the \textit{FLCN} coding regions. Significant variations in the expression of \textit{FLCN} were observed in the lung cysts of these patients, when compared with those of the patients with BHD and the controls. It was hypothesized that transcriptional regulation of \textit{FLCN} may be an important mechanism contributing to the development of lung cysts and, subsequently, PSP. The present study aimed to search for epigenetic variations in the putative promoter of \textit{FLCN} in the patients with PSP.

\textbf{Materials and methods}

\textit{Patients}. The present study was approved by the ethics committees of Nanjing University Medical School, Nanjing Chest Hospital and Taizhou Hospital of Zhejiang Province. The 71 selected patients with PSP included 69 sporadic patients and 2 patients with a family history. The patients were clinically diagnosed with PSP based on the basis of a thorax computed tomography scan and underwent surgeries for the treatment of pneumothorax at two tertiary hospitals, Taizhou Hospital of Zhejiang Province and Nanjing Chest Hospital. The patients were enrolled for the present study since they exhibited \textit{FLCN}-like multiple lung cysts, however, exhibited no mutations in the \textit{FLCN} coding region. A thorough screen for skin and renal abnormalities was performed by cutaneous examination and abdominal ultrasonography, respectively. Peripheral blood samples and tissue samples derived from the clinically resected lung lesions were collected. The control group included 11 morphologically normal lung tissues, which were obtained from patients with stage I non-small cell lung carcinomas that underwent lobectomy. These samples were obtained at least 5 cm from the tumor locus. Written informed consent was obtained from all patients involved in the present study.

\textit{DNA sequencing}. Genomic DNA was extracted using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). The \textit{FLCN} exons and flanking intron regions were sequenced, as previously reported (1). The PCR products were amplified using the BigDye Terminator kit (Applied Biosystems Life Technologies, Foster City, CA, USA) and sequenced on an ABI 3130 Genetic Analyzer (Applied Biosystems Life Technologies). The data were analyzed by referring to the reference sequence (NM_144997.5) obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/gene/201163# reference-sequences).

\textit{Haplotype analysis}. Haplotype analysis was performed in the family of patient F260. A total of 16 individuals were genotyped, among which 7 were affected and 9 were unaffected. Eight microsatellite markers were used, which spanned an 11.4-cM distance flanking the \textit{FLCN} locus on chromosome 17, including D17S799, D17S921, D17S122, D17S1857, D17S740, D17S2196, D17S2187 and D17S798. Haplotypes were determined from the genotype order in which the least number of recombinants occurred.

\textit{Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)}. The total RNA was extracted from the lung tissues using an RNaseasy Mini kit (Qiagen). The RNA was reverse transcribed into cDNA using PrimeScript RT reagent kit (Takara Bio, Inc., Dalian, China). The mRNA expression of \textit{FLCN} was determined using FastStart TaqMan Probe Master (Roche, Basel, Switzerland) on a StepOne Real-Time PCR system (Applied Biosystems Life Technologies). Probe #63 (cat. no. 4688627001; Roche) and the intron-spanning primer pair, Forward: 5'-GGACCACTGCTCGTCTG-3' and reverse: 5'-GTTGAACTTAAAGGACACTTCTA-3', were selected for \textit{FLCN}. \textit{GAPDH} was selected as a control gene for normalization. The data were analyzed using the 2-ΔΔCt method (22). Each sample was run in triplicate and all reactions were performed twice.

\textit{Western blot analysis}. Lung cyst samples were homogenized using a dounce homogenizer (Corning, Shanghai, China) and suspended in ice cold lysis buffer containing protease inhibitors (Beyotime Institute of Biotechnology, Shanghai, China). The homogenates were centrifuged at 13,000 x g for 20 min at 4°C. The protein concentrations of the supernatants were quantitated using a BCA Protein Assay kit (Beyotime Institute of Biotechnology). A total of 100 mg protein was separated by 12% SDS-PAGE and transferred onto immobilon membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% non-fat milk in Tris-buffered saline with 0.05% Tween20 (TBST; Millipore) at room temperature and then washed with TBST for 15 min three times. The membranes were incubated overnight at 4°C with the primary antibodies rabbit anti-\textit{FLCN} (D14G9) monoclonal antibody (mAb) (cat. no. 3697; Cell Signaling Technology, Beverly, MA, USA; 1:1,000 dilution) and rabbit β-actin (13E5) mAb (cat. no. 4970; Cell Signaling Technology; 1:1,000 dilution). Blots were washed in TBST for 15 min three times and subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (cat. no. ab6721; Abcam, Cambridge, UK; 1:3,000 dilution). Immunoreactive bands were detected using an eECL western blot kit (CWBio, Beijing, China). The images of the bands were captured using a digital G-box chemiluminescent imaging system (Syngene, Frederick, MD, USA). Each sample was analyzed at least twice.

\textit{Methylation analysis}. The CpG island region in the \textit{FLCN} gene was predicted using the University of California Santa
Cruz (UCSC) genome browser (http://genome.ucsc.edu) and CpGplot software (version EMBOSS 6.6.0.0; http://www.ebi.ac.uk). A panel of 13 pyrosequencing assays were performed to quantify the methylation value of 69 CpG islands for each sample. The primers for amplification and pyrosequencing were designed using PyroMark assay design software v.2.0 (Qiagen) (Table I). A total of 500 ng DNA sample was treated with EZ DNA methylation-Gold kit (Zymo Research, Orange, CA, USA), and the converted DNA was amplified using a PyroMark PCR kit (Qiagen), according to the manufacturer's instruction. The PCR products were pyrosequenced and the data were analyzed on a PyroMark Q96 ID (Qiagen). A value of 6% was set as a convincing absolute threshold for methylation, according to manufacturer's instructions and suggestions from a previous study (23).

Combined bisulphite restriction analysis (COBRA) was further performed, as described previously (24). A 281 bp sequence within the CpG islands, including 26 CpGs (CpG28-53), were amplified by semi-nested PCRs with no bias towards methylated or unmethylated templates. The PCR products were treated with the restriction endonucleases, TaqI (Promega, Madison, WI, USA) and BstUI (BioLabs, Ipswich, MA, USA), and were subsequently separated by gel electrophoresis to detect the digestion products. The universal methylated human DNA standard (Zymo Research) was used as a positive control.

Statistical analysis. All statistical analyses were performed using the SPSS statistics 17.0 software (SPSS, Inc., Chicago, IL, USA). For mRNA expression, the statistical significance was determined using unpaired, two-tailed Student's t-test. For the DNA methylation status, the statistical significance was evaluated using the Mann-Whitney U-test. P<0.05 was considered to indicate a statistically significant difference.

Results

**FLCN irregulation is observed in lung cysts of PSP.** To determine whether the FLCN mutation-like lung phenotype was associated with an alteration in the expression of FLCN, the mRNA expression levels of FLCN in lung cyst lesions from the 71 patients with PSP were determined. A wide-spread distribution of the mRNA expression levels of FLCN was observed, when compared with those of the BHD and control groups (Table II; Fig. 1A). As seen in Fig. 1A, the mRNA expression levels of FLCN in lung cyst lesions from the
Hypoid insufficiency of FLCN is pathogenic. To investigate the mechanism of FLCN irregulation in the patients with PSP, the sequence variations in the cis-regulatory region of FLCN were initially identified. A 1.6 kb upstream fragment, including the 5'-flank (650 bp), non-coding exon 1 (228 bp) and partial intron 1 (700 bp) of FLCN, was sequenced. No sequence mutation was identified in any of the 71 patients. A total of three common single nucleotide polymorphisms were confirmed, including rs1708629, rs1736209 and rs1736208.

The samples were subsequently investigated by MLPA analysis for large deletions/duplications. An FLCN intragenic deletion was detected in patient F260, with a positive family history. Pedigree and haplotype analysis revealed that the deletion co-segregated among the affected individuals in the family (Fig. 2A). PCR amplification and bidirectional sequencing of the junction fragment revealed a 7,543 bp deletion, including the non-coding exons 1-3 and a 1.3 kb upstream sequence (Fig. 2B). The deletion resulted in the removal of the putative FLCN promoter region. All family members harboring the deletion exhibited multiple lung cysts, basally located or randomly distributed on the lung (Fig. 2C-E), and two experienced pneumothorax. These phenotypic features were similar with the clinical manifestations of patients with pathogenic FLCN mutations.

To confirm that this heterozygous deletion disrupted the transcription of the mutant allele of FLCN, the expression of FLCN in patient F260 was determined. About a 50% reduction in the level of FLCN was observed in lung cyst lesions from patient F260 at the mRNA (47.4%) and protein (43.5%) expression levels, as compared with those of the controls (Fig. 2F and G). These results suggested that FLCN insufficiency caused by the heterozygous FLCN promoter deletion is pathogenic in the development of lung cysts.

Smallest region of overlap (SRO) for the FLCN promoter deletions contains CpG islands. The FLCN promoter appeared to be a recombination hotspot, in which several large deletions were identified in families with BHD (19). Within the SRO (1,893 bp) for all deletions, a CpG-enriched sequence was predicted by the UCSC genome browser and CpGPlot software (Fig. 3A). The CpG islands, located -628 to +335 of the transcription starting site, encompass the 5'-flank, exon 1 and partial intron 1, with 72 CpG loci (Fig. 3B). This region is predicted to contain multiple putative regulatory elements, including Sp1, AP-2, GCF and Early-Seq1 (Promoter Scan software, http://www-bimas.cit.nih.gov). It was speculated that the methylation status of the CpG islands may potentially affect the binding of transcription factors and consequently result in the alteration of the expression of FLCN.

Promoter methylation is not associated with FLCN irregulation. The methylation status of the FLCN promoter in 70 patients and 11 controls was quantitatively analyzed by pyrosequencing. No statistical difference in the methylation pattern of the FLCN CpG islands was observed between different groups of patients with PSP and the controls. Dense methylation across the CpG islands was not observed in any sample analyzed, neither patients nor the controls (Fig. 3C). For each sample, >95% of the CpG loci were unmethylated. Low methylation values, often <15%, at seldom individual loci (CpG38,49,53) were detected in a few samples, however, none were demonstrated to be associated with the expression of FLCN. The methylated CpGs were often separated
by neighboring unmethylated CpGs. In short, no correlation between methylation levels and the expression of **FLCN** was established.

To further confirm the pyrosequencing results, COBRA methylation analysis of the CpG islands was performed in all patients and controls. The 281 bp amplified region (CpG28-53) included two **Taq** I sites (tcga) and **Bst** UI sites (cgcg), however, following bisulphite treatment, no digestion products were observed in any sample. The absence of the digestion products of restriction endonucleases demonstrated that the originally unmethylated sites were lost due to bisulphite conversion. All the CpG loci analyzed were unmethylated.

**Discussion**

**PSP** is a clinical hallmark of BHD syndrome, since lung cysts/spontaneous pneumothorax are the most frequent and the initial presenting manifestation compared with the skin or renal features (10-12). Up to 80% of patients with BHD were identified to exhibit multiple lung cysts, and 24% experienced pneumothorax. Our previous study demonstrated that ~10% of PSP cases are caused by germline mutations in the **FLCN** gene (2). However, more sporadic PSP cases with **FLCN**-like lung cysts, however, no **FLCN** mutations, were observed. Whether an **FLCN**-associated mechanism was involved in these cases, and in addition, whether a mechanism other than genetic defect, including epigenetic alteration, is responsible for the development of **FLCN**-like lung cysts are interesting and worthy of further investigations.

Previous studies of BHD are predominantly focused on the germline mutations in the **FLCN** coding region. In the present study, a heterozygous **FLCN** promoter deletion was identified in a family with PSP exhibiting characteristic **FLCN**-like lung cysts. A reduced expression of **FLCN** was observed in the lesion tissues, consistent with a previous study, which assessed the **FLCN** promoter function in vitro (19). The present study provided further evidence to support the notion that the downregulation of **FLCN** is pathogenic in lung cyst formation. Epigenetic mechanisms have long been associated with gene regulation and human disease (25). In renal tumors, the involvement of **FLCN** promoter methylation has been hypothesized (20,21), however, inconsistent observations were reported (24,26). No previous studies have investigated the association between **FLCN** promoter methylation and the development of lung cysts. The present study observed significant variability in the expression of **FLCN** in the lung lesions of patients with non-**FLCN** mutant sporadic PSP, however, demonstrated no evidence for the association of **FLCN** promoter methylation with these cases. The lack of **FLCN** promoter methylation in patients with PSP with **FLCN**-like lung cysts is a valuable observation, which provided evidence to refocus our future research. Future studies targeting both genetic and epigenetic mechanisms are required to elucidate the molecular nature of **FLCN** irregulation in PSP.
Figure 3. Methylation status of the \textit{FLCN} promoter in patients with PSP. (A) The genomic location of the \textit{FLCN} exons 1-3, CpG Islands, deletions identified and the smallest region of overlap on chromosome 17p11.2. The information refers to the GRCh37/hg19 annotation in the UCSC and GenBank database. Families C, D and E were previously reported (19). *Family 260 was identified in the present study. (B) The CpG Islands encompass the 5' flank, exon 1 and partial intron 1 of \textit{FLCN}, and includes 72 CpG loci. (C) The methylation status of 70 patients and 11 controls was assessed. Each line represents a subject analyzed by pyrosequencing, and each row represents a CpG locus. The black boxes indicate a methylated CpG island (value, ≥6%) and white boxes indicate unmethylated CpG islands. No significant difference in the methylation of the \textit{FLCN} promoter was identified between the patients and the controls. (D) Representative data of \textit{Taq}I and \textit{Bst}UI restriction analysis of the COBRA PCR products in the patients and the controls. The absence of digestion products indicated that the originally unmethylated restriction sites of \textit{Taq}I (tca) and \textit{Bst}UI (cgcg) were lost due to bisulphite conversion. All samples tested were, therefore, unmethylated. The positive control was the universal methylated human DNA standard. PCR, polymerase chain reaction; PSP, primary spontaneous pneumothorax; U, PCR products without digestion; T, PCR products digested by \textit{Taq}I; B: PCR products digested by \textit{Bst}UI.
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