Pathology International

Pathology International 2009; **59**: 720–728 doi:10.1111/j.1440-1827.2009.02434.x

Original Article

Lung cysts in Birt-Hogg-Dubé syndrome: Histopathological characteristics and aberrant sequence repeats

Shunsuke Koga,[1,2] Mitsuko Furuya,[3] Yoko Takahashi,[1] Reiko Tanaka,[4] Atsushi Yamaguchi,[5] Kazuhiro Yasufuku,[6] Kenzo Hiroshima,[2] Masatoshi Kurihara,[7] Ichiro Yoshino,[6] Ichiro Aoki[3] and Yukio Nakatani[1,2]

[1]*Department of Pathology, Chiba University Hospital, Departments of* [2]*Diagnostic Pathology,* [5]*Neurobiology and* [6]*Thoracic Surgery, Chiba University Graduate School of Medicine,* [4]*Medical Mycology Research Center, Chiba University, Chiba,* [3]*Department of Pathology, Yokohama City University, Graduate School of Medicine, Yokohama and* [7]*Pneumothorax Center, Nissan Tamagawa Hospital, Tokyo, Japan*

Birt-Hogg-Dubé (BHD) syndrome is a rare disorder inherited in an autosomal dominant manner. The affected patients are predisposed to cutaneous fibrofolliculomas, renal cell tumors and lung cysts with recurrent pneumothorax. Contrary to neoplastic events in the skin and the kidney, the lung cysts have frequently been confused with non-neoplastic changes such as blebs or bullae. Herein is reported a case of multiple lung cysts associated with BHD syndrome. Detailed histopathological characteristics of the lesion are also given. The lung cysts were closely associated with the peripheral interlobular septum, visceral pleura or septal-pleural junctional region. These cysts were partly abutting alveolar structures, and lined by a layer of alveolar epithelium. These unique microscopic features supported the notion that the BHD lung lesions are distinct from other types of bullous changes. Genomic DNA analysis indicated an aberrant sequence repeat that caused frameshift mutation. Immunohistochemistry showed the localization of folliculin, the BHD gene-encoding protein, in macrophages and epithelial cells in the patient's and normal control's lungs. Haploinsufficiency of folliculin may cause deranged alveolar development, leading to the aberrant cystic alveolar formation. The unique mutation patterns of abnormal sequence repeats in patients with BHD syndrome are also reviewed.

Key words: alveolar cysts, Birt-Hogg-Dubé syndrome, pneumothorax, sequence repeat

Correspondence: Mitsuko Furuya, MD, PhD, Department of Pathology, Yokohama City University Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan. Email: mfuruya@yokohama-cu.ac.jp

Received 2 April 2009. Accepted for publication 8 June 2009.

Birt-Hogg-Dubé (BHD) syndrome is an autosomal-dominant hereditary disorder, and the responsible gene for this syndrome is mapped in the region of chromosome 17p11.2.[1–3] The affected family members with BHD gene mutation have a risk of developing cutaneous fibrofolliculomas of the head and neck, renal cell tumors including chromophobe and hybrid oncocytic/chromophobe renal cell carcinomas, and multiple lung cysts that often lead to recurrent pneumothorax.[3–8] In addition to the triad listed here, a possible association between BHD gene mutation and colorectal cancer has also been suggested. The examined sample group, however, is not large enough to allow a definitive conclusion, and the increased risk of colonic neoplasm is a subject for future study.[2,9–12]

Based on the neoplastic changes in the skin and in the kidney that characterize BHD syndrome, the BHD gene is currently regarded as a tumor-suppressor gene.[6] The detailed mechanism of tumorigenesis in these organs triggered by BHD gene mutation, however, is not well understood at present. Northern blot indicated that the BHD gene was detectable in several organs including the brain, the heart, the kidney and the lung, whereas it was very weak or undetectable in the muscle and the colon.[3] Histology using *in situ* hybridization showed that BHD mRNA was detectable at the spinous layer of epidermis and appendages in the skin, distal tubules in the kidney, macrophages and pneumocytes as well as stromal fibroblasts in the lung.[13] Among patients with BHD pedigrees, fibrofolliculomas typically develop at 20–40 years of age, and loss of heterozygosity (LOH) was not evident in the skin lesions.[14] In contrast, renal cell tumors are detected generally in middle-aged people.[10,12,14,15] This may be explained in part by the fact that somatic events such as LOH and second-hit mutations of BHD gene are required for the progression of renal cell tumors.[16] These clinical and

pathological data suggest that the families with germline mutation of BHD gene allele are potentially affected by typical symptom of the skin in their youth, and that the risk of renal cell carcinomas increases as they become middle-aged.

Contrary to the neoplastic processes in the skin and the kidney, no tumorigenic changes have been reported in the lung among BHD pedigree subjects. At present, the association of BHD gene mutation with pneumothorax is poorly understood. With regard to multiple cystic changes in the lung, a few reports have suggested that BHD gene-encoding protein folliculin (FLCN) may play a unique role in the lung that is different from the tumor suppression presumed in the skin and the kidney.[5,17] Very limited information is currently available about histopathological clues for differential diagnosis between BHD syndrome-associated lung cysts and other bullous changes. The mechanism of cystic changes in BHD patients is entirely unknown.[5,18]

In the present study we investigated lung cysts in a patient who had no familial history of pneumothorax and renal tumors. Distinctive histopathology of the resected lung tissue indicated that the possible mutation of BHD gene should be investigated. Genomic DNA analysis identified an aberrantly repeated sequence tag in BHD gene, confirming that the cysts formation was attributable to BHD syndrome. These lung cysts were shown to be clearly distinctive on histopathology from those of other types of bullous changes. We also demonstrated FLCN localization in the lung that had not been investigated at the protein level previously. In addition, reported aberrant sequence repeat sites that are distributed to several exons of BHD gene among BHD pedigree subjects were reviewed.

MATERIALS AND METHODS

Samples

The resected lung tissue was fixed with 20% formalin and embedded in paraffin. Written informed consent was obtained from the present patient and her parents. HE and EVG staining was done. Four normal lung tissues from samples resected for lung cancer ($n = 2$), thymoma ($n = 1$) and fungal infection ($n = 1$) were used as a positive control for immunohistochemistry.

Immunostaining

Rabbit polyclonal antibodies against full-length FLCN (sc-25777, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and prosurfactant protein C (proSP-C, Millipore, Bedford, MA, USA), and mouse monoclonal antibody against human cytokeratin (clone CAM5.2, BD Biosciences, Mountain View, CA, USA) were used. Immunohistochemistry was done using Envision kit (Dako, Carpinteria, CA, USA) and autoclave antigen retrieval. Working dilutions of the antibodies were 1:50 for FLCN, 1:100 for cytokeratin and 1:1000 for proSP-C.

DNA isolation

Written informed consent was obtained from the patient and her parents for the analysis of BHD genes prior to this study. The study was approved by the Institutional Review Board (IRB) of Chiba University School of Medicine. DNA from peripheral blood leukocytes was obtained using LabPass Blood Mini kit (Cosmo Genetech, Seoul, Korea) according to the manufacturer's instructions.

Direct sequencing

Fourteen exons of BHD gene were amplified on polymerase chain reaction (PCR) using the primers described previously.[3] PCR conditions were as follows: 95°C for 5 min, 35 cycles at 96°C for 5 s, 60°C for 5 s, 68°C for 3 s, with an extension step of 1 min at 72°C at the end of the last cycle. After purification, DNA was labeled with Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Cleveland OH, USA) and DNA sequencing was done using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). In the region where nucleotide alteration was suggested, the PCR products were subcloned (TA Cloning Kit, Invitrogen, San Diego, CA, USA) and then sequenced to clarify the mutation pattern.

Single-strand conformation polymorphism

A total of 2 μl of PCR products and formamid were incubated at 97°C for 5 min, then loaded onto microgel and electrophoresed for 10 min using micro TGGE (Taitec, Saitama, Japan).

Plasmid constructs and transfection

The cDNA fragments encoding full-length FLCN were amplified by PCR from human cDNA library and sub-cloned into pHA-C1 mammalian expression vector containing hemagglutinin (HA) epitope tag.[19] All constructs were verified by DNA sequencing. Cos7 cells, HeLa cells and HEK293 cells were cultured and transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Western blot

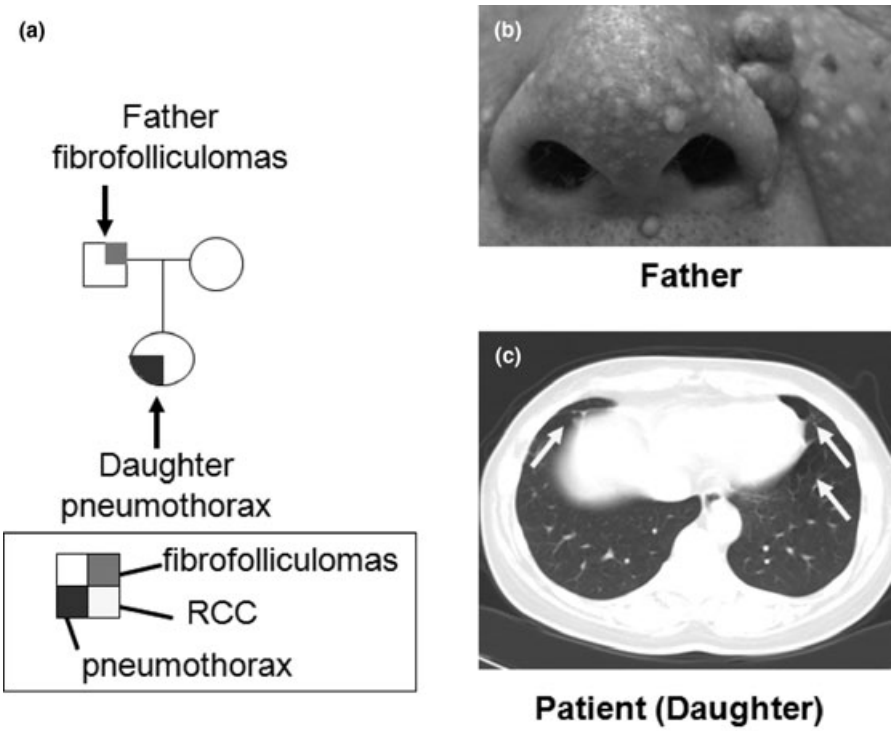
The same amount of proteins of cell lysates were electrophoresed on 12.5% sodium dodecylsufate–PAGE and transferred to a PVDF membrane (Millipore). Horseradish

peroxidase-conjugated goat anti-rabbit IgG (1:2500) was used as the secondary antibody. Bands were detected using an enhanced chemiluminescence system, according to the Hybond ECL protocol (GE Healthcare, Buckinghamshire, UK).

RESULTS

Clinical and histopathological findings

A 41-year-old Japanese woman was admitted to Chiba University Hospital for investigation of pneumothorax that had occurred twice (7 years previously and a few months previously). The patient was a non-smoker and did not have specific diseases such as endometriosis or α-1-antitrypsin deficiency. The patient's parents did not have a history of pneumothorax or renal cell tumors but it was later found that the patient's father had skin eruptions, that is, fibrofolliculomas (Fig. 1a,b). CT of the patient's chest demonstrated bilateral pulmonary cysts localized predominantly at the subpleural regions of lower lobes and perimediastinal regions (Fig. 1c). The patient subsequently underwent pulmonary wedge resection to prevent repeated pneumothorax and enable pathological diagnosis.



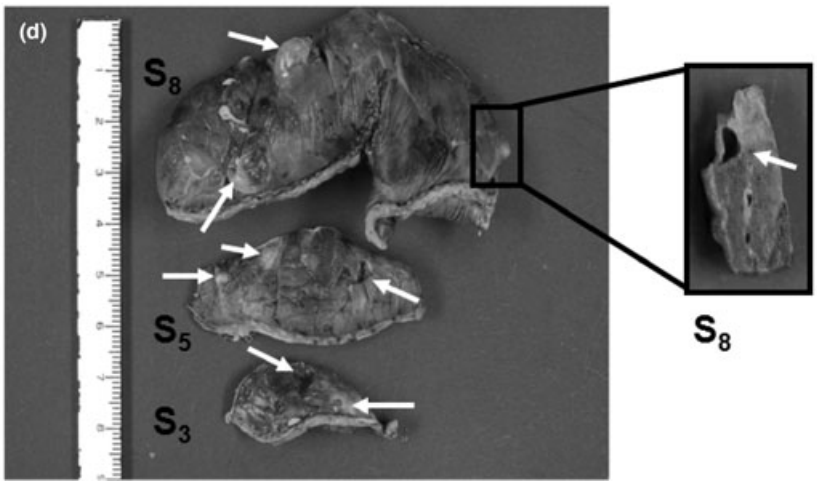


Figure 1 Inheritance of Birt-Hogg-Dubé gene mutation and macroscopic findings. (**a**) The father had fibrofolliculomas in the head and neck. He had no episode of pneumothorax. The daughter had pneumothorax twice but no fibrofolliculomas. (**b**) Fibrofolliculomas detected on the father's face. (**c**) CT of the daughter's chest after treatment of the latest pneumothorax, indicating predisposing condition to further rupture of remaining cysts (arrows). (**d**) Removed multiple cystic lesions localized in pleural to subpleural areas (arrows).

The submitted specimen consisted of three wedge-resected tissues from cystic lesions of the right pulmonary lobes. Macroscopically, several cysts up to 1cm in diameter were found in each tissue, mostly in the pleural and subpleural regions (Fig. 1d, arrows). On microscopy the cysts were found to be located in the vicinity of the interlobular septa, visceral pleura, and junctional regions between the interlobular septum and visceral pleura (Fig. 2a,d). Close examination indicated that each cyst was incorporated with interstitial stroma of the interlobular septum and/or pleura in part, and with normal alveolar structures in the other part (Fig. 2b,e). EVG staining identified the elastic framework that demarcated the cyst wall from the stroma of interlobular septum (Fig. 2f, arrows). The elastic layer of the cyst abutting normal alveoli joined in the framework of the normal alveolar wall (Fig. 2f, arrowheads). The inner surface of the cyst was lined by proSP-C- and cytokeratin-positive pneumocytes (Fig. 2c,g). Although most cysts were unilocular, one cyst had double cystic spaces resembling giant alveoli (Fig. 2d–g). Some cysts had protrusion of veins into the cystic space. Neither active stromal cell proliferation nor inflammatory cell infiltration was observed in the vicinity of the cystic lesions, although focal exudative reaction was noted around a ruptured cyst. A few non-specific blebs and dilated air spaces were also seen, which were thought to be secondary changes. Together with the abnormal location of the cysts, microscopic features such as epithelial lining and absence of stromal proliferation suggested that the lesions were different from emphysematous bullae and subpleural apical blebs. There was no evidence of lymphangioleiomyomatosis or endometriosis. Although the patient did not have a familial history of pneumothorax, BHD syndrome was suspected as the underlying disorder.

Aberrant sequence repeat in exon 12

After obtaining informed consent and IRB approval, we performed genomic DNA analysis of BHD gene on PCR. The bands of each PCR product from exon 1 to exon 14 were of the predicted size (data not shown), indicating that massive gene deletion was unlikely. Next we performed direct sequencing of all 14 exons, and found that exon 12 contained a possible mutational change. Cloning of the PCR products showed an insertion of seven nucleotides in exon 12 (nt 1795 ins CCACCCT) in three of five clones. The affected site had a repeat of CCACCCT in the control (Fig. 3a). In the patient's BHD gene allele, a three-repeat of CCACCCT was demonstrated (Fig. 3b). This mutation is predicted to cause a frameshift, which probably results in FLCN truncation at 16 nucleotides downstream (Fig. 3b). In PCR–single-strand conformation polymorphism, products of exon 12 from the patient's DNA showed a specific band that was not seen in the corresponding control of exon 12, confirming the presence of gene mutation in this region (Fig. 3c, arrow).

After the diagnosis of BHD syndrome-associated lung cysts, the patient's parents agreed to have genomic DNA examination. The same mutation pattern was detected in exon 12 of the father's DNA (data not shown). It was concluded that this mutation was inherited by the daughter from the father. In this family, ins CCACCCT of exon 12 generated different phenotypic disorders; that is, fibrofolliculomas in the father and lung cysts with pneumothorax in the daughter (Fig. 1a). Although we could not exclude the possibility that the father had lung cysts, he did not agree to have radiological examination.

Expression of FLCN in the lung

To clarify whether FLCN, the protein encoded by BHD gene, is detectable in the lung, immunostaining was performed. According to a previous study using *in situ* hybridization, BHD mRNA was shown to be expressed in alveolar macrophages, stromal fibroblasts and pneumocytes of the lung.[13] First, we confirmed the sensitivity and specificity of anti-FLCN antibody using HA-tagged FLCN transfected cells. Western blot confirmed that the antibody recognized full-length FLCN at an appropriate molecular weight (Supplement Fig. S1a). On double cytostaining, HA-positive transfected cells were stained for FLCN in a specific manner (Supplement Fig. S1b). We concluded that this antibody was available for analysis of FLCN localization in human specimens on immunohistochemistry.

In normal lungs ($n = 4$), FLCN was shown to be positive for bronchiolar epithelia (Fig. 4a), alveolar macrophages (Fig. 4b), and some type II pneumocytes (Fig. 4c, arrows). The same staining pattern was demonstrated in all four normal lung tissues obtained from different patients. These results were consistent with the data obtained using *in situ* hybridization.[13] Some stromal cells were also positive in the present study, and double immunofluorescence staining suggested that these cells were not endothelial cells but some other type of mesenchymal cells (data not shown). Although the previous work did not describe whether bronchiolar epithelia expressed BHD mRNA or not,[13] the present results demonstrated that FLCN was strongly localized in these cells. With regard to the BHD lung, the normal-looking areas showed positive immunostaining in bronchiolar epithelia (Fig. 4d), alveolar macrophages (Fig. 4e, arrowhead), and some type II pneumocytes (Fig. 4e, arrows). In the cyst lesions the pneumocytes that lined the inner surface of cysts were also immunostained for FLCN (Fig. 4f, arrows). The results suggested that FLCN is also present in the lungs of BHD patients, with no apparent difference from the control lung at least in the normal-looking regions.

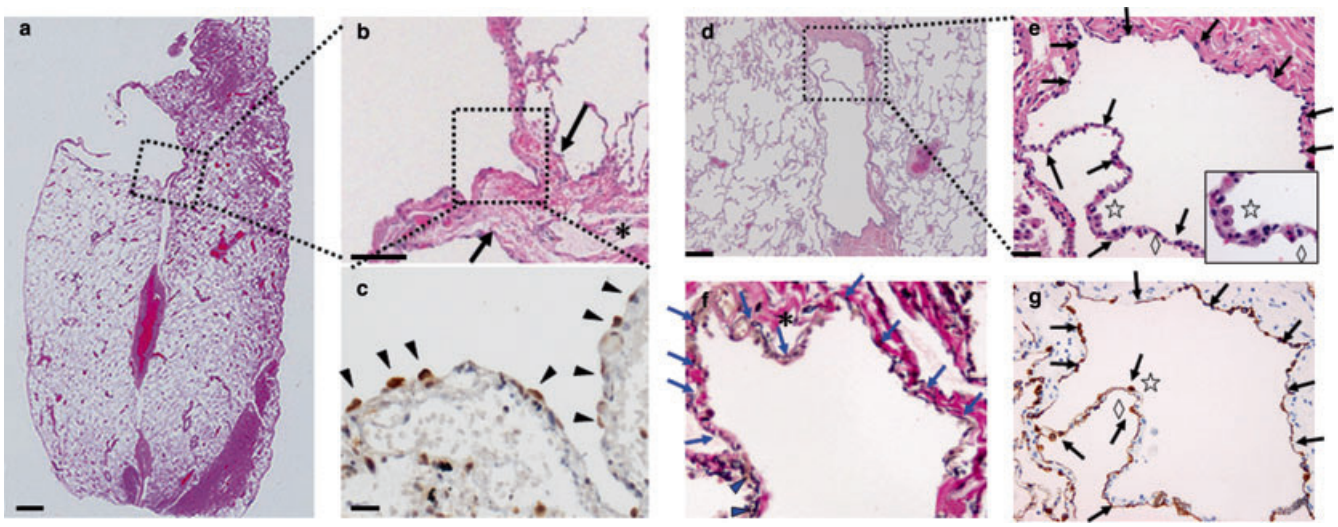


Figure 2 Microscopic feature of Birt-Hogg-Dubé syndrome-associated pulmonary lesions. (**a**) HE staining of the lung. A cystic lesion was localized in the interlobular septal-pleural junction region (bar, 1 mm). (**b**) Higher magnification shows that a part of the cyst is enveloped by interstitial tissue of an interlobular septum (arrows;). Interlobular septum (*). Bar, 500 μm. (**c**) Immunohistochemistry of prosurfactant protein C (proSP-C) in a serial section. Note that the inner surface of the cyst is lined by pneumocytes (bar, 20 μm). (**d**) Double-spaced cyst partially incorporated within an interlobular septum (bar, 200 μm). (**e**) Higher magnification of the cyst wall. The inner surfaces of upper (star) and lower (diamond) cysts are lined completely by pneumocytes, respectively (arrows; bar, 50 μm). Inset: further magnification of the septum between upper and lower cysts. The septum contains capillaries inside and is lined by pneumocytes on both surfaces. (**f**) EVG stain shows that the cyst is partially within the septum (*). The cyst itself has an ill-developed elastic layer (arrows) and it unites with alveolar elastic framework where the cyst wall is directly adjacent to alveoli (arrowheads). (**g**) Immunostaining for cytokeratin in a serial section. Epithelial-lining cells of both cysts are highlighted (arrows).

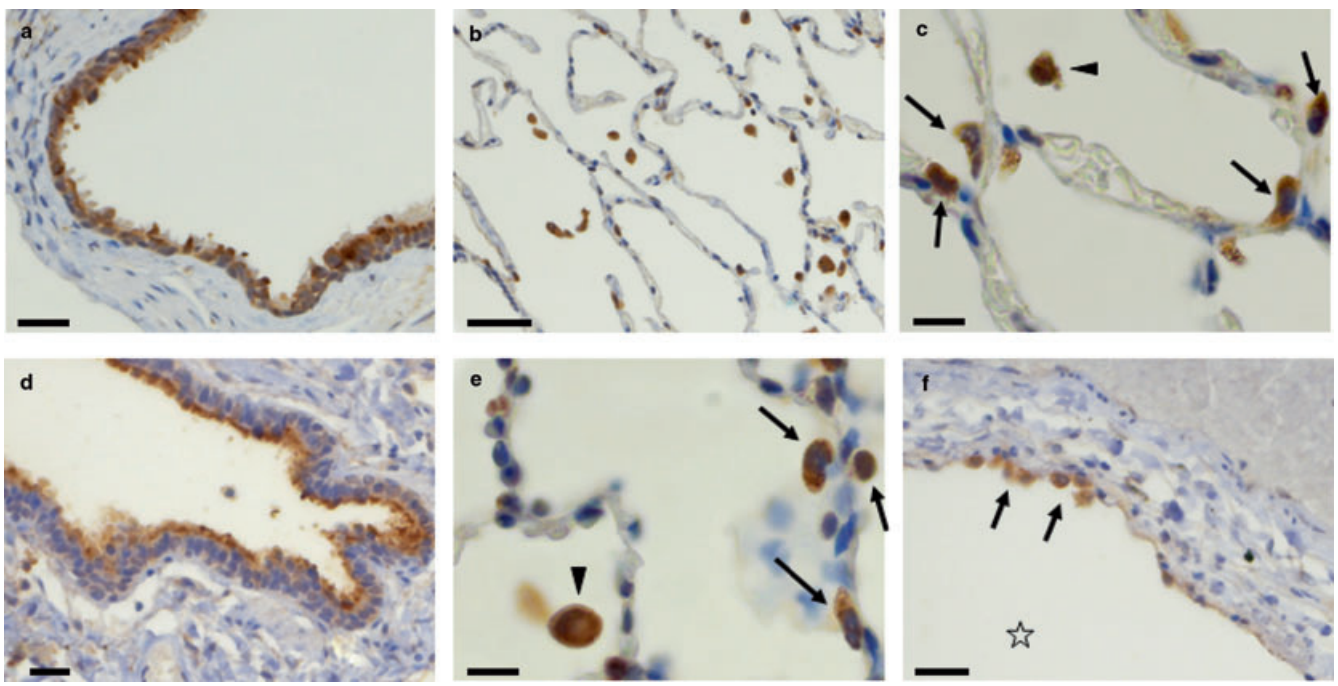


Figure 4 Immunostaining for folliculin (FLCN) in the lung. In normal lung tissues, FLCN staining is detected in (**a**) bronchiolar epithelial cells (bar, 20 μm), (**b**) alveolar macrophages (bar, 50 μm), and (**c**) an alveolar macrophage (arrowhead) and some type II pneumocytes (arrows; bar, 10 μm). In Birt-Hogg-Dubé (BHD) lung tissues, FLCN staining is detected in (**d**) bronchiolar epithelial cells (bar, 20 μm), (**e**) alveolar macrophages (arrowheads) and some type II pneumocytes (arrows; bar, 10 μm). (**f**) In the cyst wall of BHD lung, lining pneumocytes are positive for FLCN (arrows). Star, inside of the cyst. Bar, 20 μm.

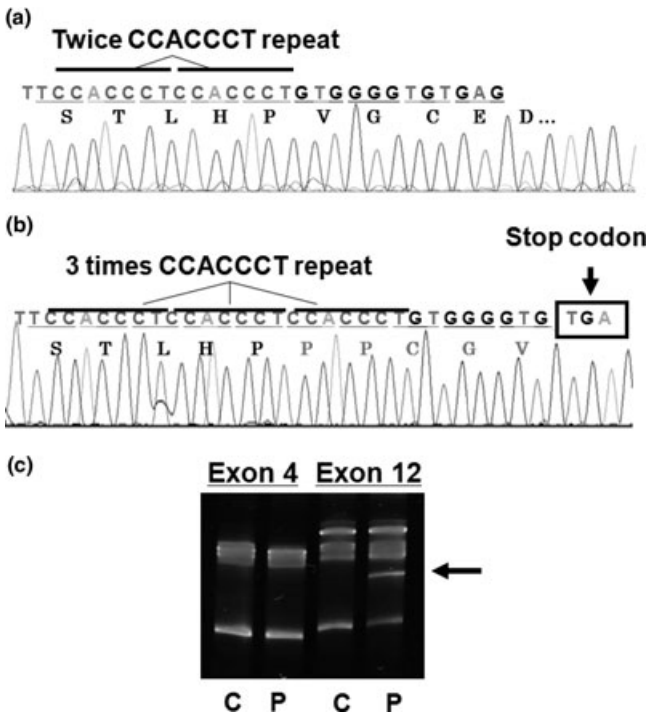


Figure 3 Mutation analysis of the Birt-Hogg-Dubé (BHD) gene. Sequence analysis of subcloned polymerase chain reaction (PCR) product: (**a**) two-CCACCCT repeat in the control's exon 12 compared to (**b**) a three-repeat in that of the patient's exon 12. (**c**) PCR–single-strand conformation polymorphism of a normal site (exon 4, left) and the mutation site (exon 12, right). An abnormal band is detected in the patient's exon 12 (arrow). C, control; P, patient.

DISCUSSION

Pneumothorax generally occurs as a consequence of subpleural blebs, bullae or congenital abnormality of the pleura.[20] Idiopathic spontaneous pneumothorax affects typically young taller and thinner male patients, whereas secondary spontaneous pneumothorax is observed in patients of various ages who have miscellaneous disorders including α-1-antitrypsin deficiency, Ehlers–Danlos syndrome, lymphangio-leiomyomatosis, endometriosis and so on.[20] In the patients who have neither familial history of pneumothorax nor skin eruption, it may be difficult to distinguish BHD syndrome-associated pneumothorax from idiopathic ones. Abnormal localization of multiple cysts in the lower lobes and perimediastinal regions on chest CT may alert physicians and pathologists to the possibility of BHD syndrome.[7]

FLCN has been shown to be involved in 5′-AMP-activated protein kinase (AMPK)-mammalian target of rapamycin (mTOR) pathways.[21] Currently, the actual function of FLCN in the lung and the development of the cysts caused by BHD gene mutation *in vivo* are poorly understood. Contrary to fibrofolliculomas and renal cell tumors, BHD syndrome-associated pulmonary cysts have been regarded as a kind of bulla or bleb and not a tumorous change.[5,18] Indeed, the arrangement of pneumocytes in the cyst wall shows neither a proliferative architecture nor cytological abnormality (Fig. 2a–g).[6] The histopathology of BHD syndrome-associated cysts, however, indicated some unique features of the epithelial-lining cysts. They are distinct from bullae that represent cystic air spaces generated by the destruction of alveolar walls, or blebs that are composed of intrapleural air collections. It should be emphasized that these cysts are lined by a layer of pneumocytes, enveloped by septal and/or pleural interstitial tissue in part, and not accompanied by reactive stromal proliferation and inflammation except in the case of rupture. They have their own delicate elastic framework beneath the epithelial layer, resembling normal alveoli in its basic structure. A small vein protrusion into the cyst lumen is also a characteristic finding, which probably corresponds to one of the unique CT findings or an intimate spatial relationship of blood vessels and pulmonary cysts reported in BHD patients.[7,22]

Based on these distinctive histopathological features of the cysts, we propose a hypothesis that the pulmonary cysts in BHD syndrome may represent an aberrant cystic alveolar formation. Deranged interaction between alveolar epithelial structures and the surrounding mesenchyme in the peripheral lobular compartment may result in the formation of abnormal cysts without stromal reaction. Some of them may further grow slowly, and cause pneumothorax. Time course pathological observation of the kidney in a Nihon rat BHD model indicated adenomatous tubular hyperplasia in the early stage of renal carcinogenesis.[23] A recent study on the transgenic mice of kidney-directed BHD inactivation using the Cre-loxP system demonstrated polycystic changes in the kidney.[24] Similarly, FLCN haploinsufficiency might trigger alveolar epithelial abnormality and cause aberrant cysts in the lung. These lesions do not show any significant proliferative activity of pneumocytes; thus their nature may be close to a non-neoplastic hamartomatous lesion rather than a true neoplasm. It is a subject for future study to clarify the molecular mechanism of the cyst formation. It is also an interesting area for future study as to whether BHD mutation is associated with the development of cystadenomatous changes or adenocarcinomas of the lung.

From a diagnostic point of view, the unique histopathological features found in the present case will be helpful for the correct diagnosis of BHD syndrome-associated lung cysts. Because non-specific blebs and bullae may occur as a secondary change in BHD-affected lungs, it is important not to miss the cysts unique to BHD syndrome on histopathology.

Cumulative studies on subjects with BHD pedigrees have contributed to our understanding of various mutation patterns that are not limited to exons but are also detected in introns.[7,12,14] Currently, it remains unclear as to whether some mutation patterns potentially cause specific disease courses,[7,8,17] or whether mutation patterns have no relevance

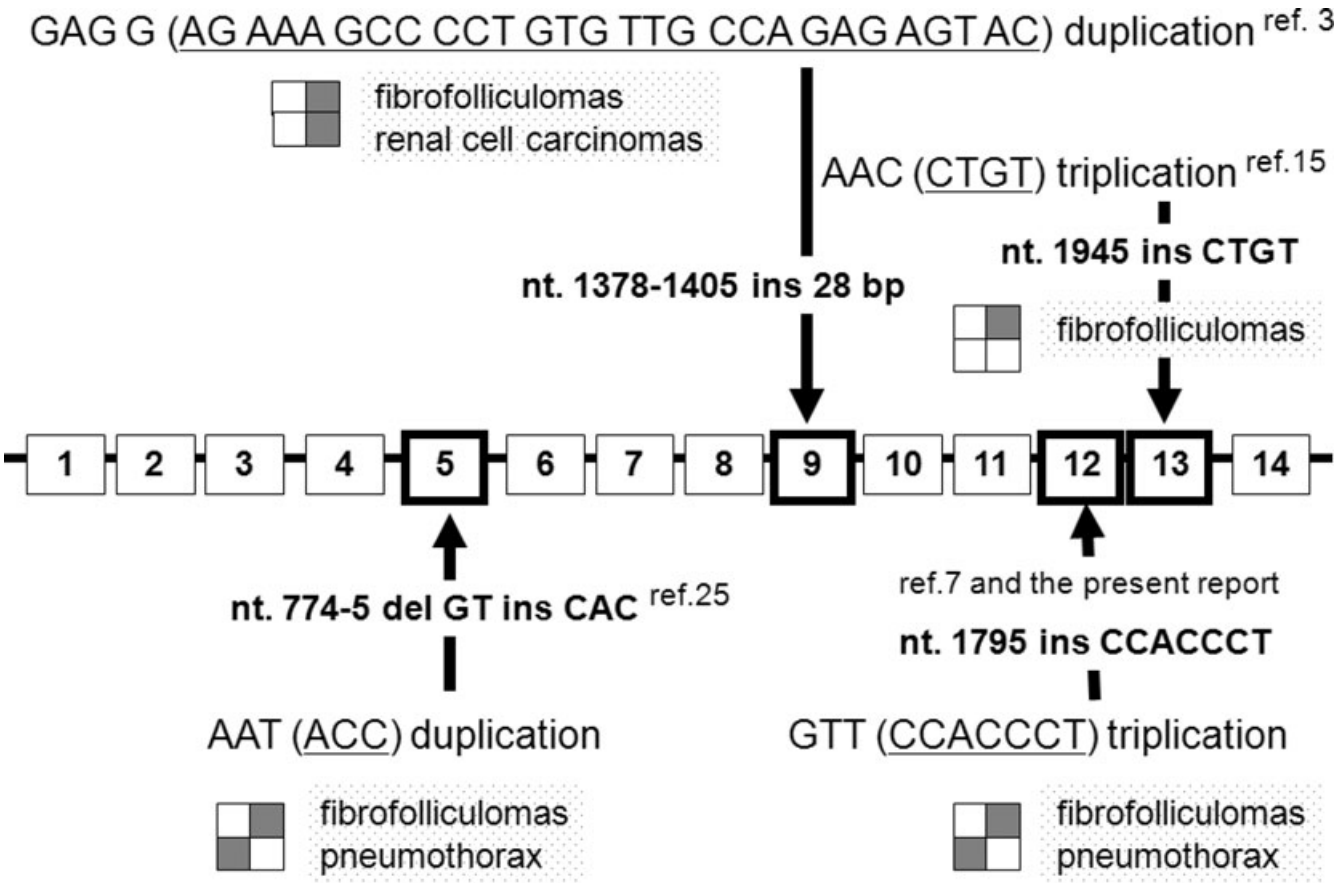


Figure 5 Sequence repeat patterns in Birt-Hogg-Dubé syndrome. In exon 5 nt 774, base substitution (del GT ins CAC) causes the duplication of ACC.[25] In exon 9 nt 1378, a 28 nucleotide insertion causes the duplication of (AGAAAGCCCCTGTGTTGCCAGAGAGTAC).[3] In exon 12 nt 1795, a seven nucleotide insertion causes the triplication of (CCACCCT)[7] (and the present study). In exon 13 nt 1945, a four nucleotide insertion causes the triplication of (CTGT).[15] del, deletion; ins, insertion.

to pathological phenotypes. Because the average age of episodes of pneumothorax in some BHD subjects is younger than that of fibrofolliculomas and renal cell tumor patients,[7,17] it is difficult to predict whether and when these BHD pedigree subjects with lung cysts are affected by the other disorders; long-term follow up is required. Most frequent patterns of BHD gene mutation include single base deletions and insertions. For example, nt 1733 in exon 11 is regarded as a hot spot in which either a deletion or an insertion of one cytosine can lead to FLCN truncation.[3,25] Contrary to such single nucleotide-associated mutations that represent this syndrome, the mutation detected in the present study involved an abnormal repeat of CCACCCT sequence tags, which caused frameshift and resulted in stop codon formation downstream. This mutation pattern has been reported recently by Gunji *et al.*, in which familial pneumothorax was observed but not fibrofolliculomas or renal cell tumors.[7] We observed that this mutation pattern caused fibrofolliculomas in the present patient's father. Among more than 50 mutation sites reported so far between exon 4 and exon 14, abnormal repeats of sequence tags may characterize genetic background of BHD syndrome in part. Review of the literature shows that there are at least four different types of aberrant repeats among BHD pedigree subjects: a duplicate of ACC in exon 5,[25] a duplicate of AGAAAGCCCCTGTGTTGCCA-GAGAGTAC in exon 9,[3] a triplicate of CCACCCT in exon 12[7] (and present case) and a triplicate of CTGT in exon 13[15] (Fig. 5). All these patterns belong to frameshift mutation and cause fibrofolliculomas among the family members. Further studies on both hazard and inherited mutation patterns will provide information on possible vulnerable sites of BHD gene and highly predisposed phenotypes. Further study is required to clarify which mutation patterns are significantly associated with life-threatening disease progressions such as metastatic renal cell carcinomas.[26]

In summary, the present study provides an insight into the lung cyst formation in BHD syndrome-associated pneumothorax. We demonstrated distinctive histopathological features of the lung lesion in BHD syndrome and localization of FLCN in human lung tissues. We also discussed several patterns of sequence repeats in affected BHD gene. Currently, little is known about the role of FLCN in normal lung

development and homeostasis. Further investigation is necessary to understand the pathological cascade of the abnormal cystic changes, unique localization of the cysts in the lung and the possibility of tumorigenesis in BHD gene mutation-harboring lungs.

ACKNOWLEDGMENTS

The authors thank members of the pathology laboratories at Chiba University Hospital and Yokohama City University Graduate School of Medicine for assistance, Drs T. Kumasaka and K. Seyama for fruitful discussion, Dr Mari Mino-Kenudson for providing relevant case material, and Mr T. Matsuzawa and Ms Y. Tsuchiya for sequencing. This work was supported by Grants in Aid for Scientific Research (M.F. and Y.N.), Hasegawa's Geriatric Research Foundation (S.K.) and Yokohama Science Education Foundation (M.F.).

REFERENCES

1 Schmidt LS, Warren MB, Nickerson ML *et al.* Birt-Hogg-Dubé syndrome, a genodermatosis associated with spontaneous pneumothorax and kidney neoplasia, maps to chromosome 17p11.2. *Am J Hum Genet* 2001; **69**: 876–82.
2 Khoo SK, Bradley M, Wong FK *et al.* Birt-Hogg-Dubé syndrome: Mapping of a novel hereditary neoplasia gene to chromosome 17p12-q11.2. *Oncogene* 2001; **20**: 5239–42.
3 Nickerson ML, Warren MB, Toro JR *et al.* Mutations in a novel gene lead to kidney tumors, lung wall defects, and benign tumors of the hair follicle in patients with the Birt-Hogg-Dubé syndrome. *Cancer Cell* 2002; **2**: 157–64.
4 Birt AR, Hogg GR, Dubé WJ. Hereditary multiple fibrofolliculomas with trichodiscomas and acrochordons. *Arch Dermatol* 1977; **113**: 1674–7.
5 Graham RB, Nolasco M, Peterlin B *et al.* Nonsense mutations in folliculin presenting as isolated familial spontaneous pneumothorax in adults. *Am J Respir Crit Care Med* 2005; **172**: 39–44.
6 Adley BP, Smith ND, Nayar R *et al.* Birt-Hogg-Dubé syndrome: Clinicopathologic findings and genetic alterations. *Arch Pathol Lab Med* 2006; **130**: 1865–70.
7 Gunji Y, Akiyoshi T, Sato T *et al.* Mutations of the Birt Hogg Dube gene in patients with multiple lung cysts and recurrent pneumothorax. *J Med Genet* 2007; **44**: 588–93.
8 Toro JR, Pautler SE, Stewart L *et al.* Lung cysts, spontaneous pneumothorax, and genetic associations in 89 families with Birt-Hogg-Dubé syndrome. *Am J Respir Crit Care Med* 2007; **175**: 1044–53.
9 Rongioletti F, Hazini R, Gianotti G *et al.* Fibrofolliculomas, tricodiscomas and acrochordons (Birt-Hogg-Dubé) associated with intestinal polyposis. *Clin Exp Dermatol* 1989; **14**: 72–4.
10 Zbar B, Alvord WG, Glenn G *et al.* Risk of renal and colonic neoplasms and spontaneous pneumothorax in the Birt-Hogg-Dube syndrome. *Cancer Epidemiol Biomarkers Prev* 2002; **11**: 393–400.
11 Vincent A, Farley M, Chan E *et al.* Birt-Hogg-Dube' syndrome: A review of the literature and the differential diagnosis of firm facial papules. *J Am Acad Dermatol* 2003; **49**: 698–705.
12 Leter EM, Koopmans AK, Gille JJ *et al.* Birt-Hogg-Dubé syndrome: Clinical and genetic studies of 20 families. *J Invest Dermatol* 2008; **128**: 45–9.
13 Warren MB, Torres-Cabala CA, Turner ML *et al.* Expression of Birt-Hogg-Dubé gene mRNA in normal and neoplastic human tissues. *Mod Pathol* 2004; **17**: 998–1011.
14 van Steensel MA, Verstraeten VL, Frank J *et al.* Novel mutations in the BHD gene and absence of loss of heterozygosity in fibrofolliculomas of Birt-Hogg-Dubé patients. *J Invest Dermatol* 2007; **127**: 588–93.
15 Schmidt LS, Nickerson ML, Warren MB *et al.* Germline BHD-mutation spectrum and phenotype analysis of a large cohort of families with Birt-Hogg-Dubé syndrome. *Am J Hum Genet* 2005; **76**: 1023–33.
16 Vocke C, Yang Y, Pavlovich CP *et al.* High frequency of somatic frameshift BHD gene mutations in Birt-Hogg-Dube'-associated renal tumors. *J Natl Cancer Inst* 2005; **97**: 931–5.
17 Painter JN, Tapanainen H, Somer M *et al.* A 4-bp deletion in the Birt-Hogg-Dubé gene (FLCN) causes dominantly inherited spontaneous pneumothorax. *Am J Hum Genet* 2005; **76**: 522–7.
18 Butnor KJ, Guinee DG Jr. Pleuropulmonary pathology of Birt-Hogg-Dubé syndrome. *Am J Surg Pathol* 2006; **30**: 395–9.
19 Endo M, Ohashi K, Mizuno K. LIM kinase and slingshot are critical for neurite extension. *J Biol Chem* 2007; **282**: 13 692–702.
20 Travis WD, Colby TV, Koss MN *et al.* Non-neoplastic disorders of the lower respiratory tract. In: King DW, Sobin LH, Stocker JT, Wagner B, eds. *Atlas of Nontumor Pathology*. Washington, DC: American Registry of Pathology and Armed Forces Institute of Pathology, 2002; 901–21.
21 Baba M, Hong SB, Sharma N *et al.* Folliculin encoded by the BHD gene interacts with a binding protein, FNIP1, and AMPK, and is involved in AMPK and mTOR signaling. *Proc Natl Acad Sci USA* 2006; **103**: 15 552–7.
22 Kumasaka T, Hayashi T, Minami K *et al.* Pathological characterization of pulmonary cysts in Birt-Hogg-Dubé syndrome. *Familial Cancer* 2008; **7**: S10.
23 Kouchi M, Okimoto K, Matsumoto I *et al.* Natural history of the Nihon (Bhd gene mutant) rat, a novel model for human Birt-Hogg-Dubé syndrome. *Virchows Arch* 2006; **448**: 463–71.
24 Baba M, Furihata M, Hong SB *et al.* Kidney-targeted Birt-Hogg-Dubé gene inactivation in a mouse model: Erk1/2 and Akt-mTOR activation, cell hyperproliferation, and polycystic kidneys. *J Natl Cancer Inst* 2008; **100**: 140–54.
25 Toro JR, Wei MH, Glenn GM *et al.* BHD mutations, clinical and molecular genetic investigations of Birt-Hogg-Dubé syndrome: A new series of 50 families and a review of published reports. *J Med Genet* 2008; **45**: 321–31.
26 Pavlovich CP, Grubb RL 3rd, Hurley K *et al.* Evaluation and management of renal tumors in the Birt-Hogg-Dube syndrome. *J Urol* 2005; **173**: 1482–6.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1 Cos 7 cells expressing full-length folliculin (FLCN). Expression plasmids coding for hemagglutinin (HA)-tagged FLCN was constructed by inserting full length cDNA into pHA-C1 vector containing the HA. (**a**) Specific single band of

FLCN is detected in transfected cell lysate (right lane) by FLCN (top) and HA (middle). (**b**) Only HA-positive cells (green, left) are stained for FLCN (red, center). Merged image with DAPI is shown (right).