Distinctive expression patterns of glycoprotein non-metastatic B and folliculin in renal tumors in patients with Birt–Hogg–Dubé syndrome

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Key words
Birt–Hogg–Dubé syndrome (BHD), familial cancer, folliculin (FLCN), glycoprotein non-metastatic B (GPNMB), renal tumor

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Funding information
Japan Society for the Promotion of Science Kakenhi (23590406). (24590408). Ministry of Health, Labor and Welfare Yokohama Foundation for Advancement of Medical Science Suzuki Hinyoki Igaku Shinko Zaidan Mitsubishi Foundation

Received July 22, 2014; Revised December 10, 2014; Accepted December 24, 2014

Cancer Sci 106 (2015) 315–323

doi: 10.1111/cas.12601

Birt–Hogg–Dubé syndrome (BHD) is an inherited disorder associated with a germline mutation of the folliculin gene (FLCN). The affected families have a high risk for developing multiple renal cell carcinomas (RCC). Diagnostic markers that distinguish between FLCN-related RCC and sporadic RCC have not been investigated, and many patients with undiagnosed BHD fail to receive proper medical care. We investigated the histopathology of 27 RCCs obtained from 18 BHD patients who were diagnosed by genetic testing. Possible somatic mutations of RCC lesions were investigated by DNA sequencing. Western blotting and immunohistochemical staining were used to compare the expression levels of FLCN and glycoprotein non-metastatic B (GPNMB) between FLCN-related RCCs and sporadic renal tumors (n = 62). The expression of GPNMB was also evaluated by quantitative RT-PCR. Histopathological analysis revealed that the most frequent histological type was chromophobe RCC (n = 12), followed by hybrid oncocytic/chromophobe tumor (n = 6). Somatic mutation analysis revealed small intragenic mutations in six cases and loss of heterozygosity in two cases. Western blot and immunostaining analyses revealed that FLCN-related RCCs showed overexpression of GPNMB and underexpression of FLCN, whereas sporadic tumors showed inverted patterns. GPNMB mRNA in FLCN-related RCCs was 23-fold more abundant than in sporadic tumors. The distinctive expression patterns of GPNMB and FLCN might identify patients with RCCs who need further work-up for BHD.

Birt–Hogg–Dubé syndrome, also called Hornstein–Knickenberg syndrome, is an inherited disorder characterized by skin fibrofolliculomas, pulmonary cysts, pneumothorax, and renal tumors.1,2 Renal cell carcinoma is the most serious and life-threatening manifestation. Since the discovery of the responsible gene, named FLCN,3 increasing numbers of BHD patients have been diagnosed by genetic testing.4–6 Bilateral and metachronous RCCs are often observed.5,6 Patients are at risk for developing independent tumors in the remaining renal tissues after surgical intervention. However, many physicians are still not aware of BHD-related symptoms, and undiagnosed patients are treated as sporadic RCC. Characterization of RCC associated with BHD is important to identify previously undiagnosed patients.

With regard to histological features, only a few studies have been published.5–7 Birt–Hogg–Dubé syndrome is morphologically characterized as a HOCT and composites of more than one histological type.5 Hybrid oncocytic/chromophobe tumor is an oncocytic neoplasm that has areas suggestive of chromophobe RCC and oncocytoma. Many large eosinophilic cells possess intracytoplasmic vacuoles.5,8 However, for a patient with an unknown genetic background, these renal tumors are virtually indistinguishable from sporadic ones solely by microscopic findings. Hybrid oncocytic/chromophobe tumor might also be found in individuals without an FLCN germline mutation.9–11 Detailed information about diagnostic markers for FLCN-related RCC is unavailable.

In the current study, we investigated the pathological features and expression of FLCN and another key molecule, GPNMB, in RCCs of BHD patients. Recently, GPNMB was identified as a downstream targeting molecule induced by FLCN inactivation.12 Here we show that RCCs with FLCN germline mutations overexpressed GPNMB and that they tended to lose the intact form of FLCN. Sporadic renal tumors showed inverted expression patterns of GPNMB and FLCN.

Materials and Methods

Samples. Eighteen patients (17 families) were enrolled in this study, providing 27 renal tumors (Tables 1,2). Written
informed consent was obtained from each patient. The study was approved by the Institutional Review Board of Yokohama City University (Yokohama, Japan). Resected tissues were fixed with 10% buffered formalin and embedded in paraffin. Hematoxylin and eosin staining was carried out for histological diagnosis. In seven tumors, RCCs and normal parts of the kidneys were snap-frozen and stored in liquid nitrogen until use. Sporadic renal tumors \( (n = 62) \) were used for comparison. The possibilities of von Hippel-Lindau disease, tuberous sclerosis complex, hereditary papillary RCC, and hereditary leiomyomatosis RCC were carefully examined and excluded in all patients by thorough medical examination and family history. The following points were checked in the studied cases in accordance with the diagnostic criteria proposed by the European BHD Consortium \(^{15} \): (i) pulmonary cysts; (ii) fibrofoliculomas; (iii) pneumotheorax; (iv) familial history of pneumotheorax, fibrofoliculoma, and renal tumor; and (v) multiple RCC or hybrid features in histology. All patients who were diagnosed with BHD by genetic testing had at least two manifestations. In the sporadic tumor groups, one patient with unilateral chromophobe RCC had pulmonary cysts and genetic testing was carried out. Other patients diagnosed with sporadic renal tumors did not have any of the manifestations listed above. Histological types were determined by two pathologists with expertise in renal tumors (N. Kuroda and Y. Nagashima).

**Antibodies.** For Western blotting of FLCN, rabbit mAb D14G9 (Cell Signaling Technology, Danvers, MA, USA) was used. For FLCN immunostaining, we used rabbit polyclonal antibody ab93196 (Abcam, Cambridge, UK) was used. For GPNMB immunostaining and Western blotting, we obtained goat polyclonal antibody AF-2550 (R&D Systems, Minneapolis, MN) was used. Mouse mAb against β-actin was purchased from Sigma (St. Louis, MO, USA).

**Immunostaining.** Four micrometer-thick paraffin sections were subjected to immunohistochemistry. For GPNMB immunostaining, sections were treated with proteinase K for 10 min at room temperature. For other antibodies, sections were autoclaved at 121°C for 15 min. The sections were treated with the diluted antibodies at 4°C overnight. Working dilutions were 1:200 for GPNMB and 1:50 for anti-FLCN antibody (ab93196). The FLCN immunostaining patterns were scored as follows. Tissues lacking staining or with ≤5% positive tumor cells regardless of cytoplasmic or nuclear pattern were termed “negative” (−). Weak to strong cytoplasmic staining without nuclear staining in ≥5% tumor cells was categorized as “cytoplasmic”. Weak to strong nuclear staining regardless of cytoplasmic staining in ≥5% tumor cells was termed “nuclear”. The GPNMB immunostaining patterns were scored as follows: no staining or ≤5% positive tumor cells was categorized as “negative” (−); occasional weak staining in ≥5% tumor cells was categorized as (+); and moderate or intense staining in ≥5% tumor cells was categorized as (++).

**DNA isolation.** DNA from peripheral blood leukocytes was obtained using the LabPass Blood Mini kit (Cosmo Genetech, Seoul, Korea), and DNA from renal tissue was obtained using

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### Table 1. Summary of clinical information of patients with Birt–Hogg–Dubé syndrome (BHD)

| Family no. | Gender | Age, years | No. of RCC | Treatments | Other findings | Prognosis (follow-up period, months) |
|------------|--------|------------|------------|------------|---------------|-------------------------------------|
| BHD1       | Female | 46         | 1          | Nx         | PTX, PC       | NED (30)                            |
| BHD2 (proband) | Female | 69         | 2          | Rt. Nx     | Skin tumors, PC | NED (54)                            |
| BHD2 (daughter) | Female | 46         | 1          | Lt. NSS    | PC            | NED (42)                            |
| BHD3       | Male   | 61         | 1          | Nx, VATS   | PTX, PC       | Lung metastasis                    |
|            |        |            |            |            |               | NED (42)                            |
| BHD4       | Male   | 67         | 3          | Lt. Nx     | Skin tumors, PC, Adrenal adenoma | NED (30)                            |
| BHD5       | Male   | 54         | >5         | Lt. Nx, Rt. NSS | PC            | NED (27)                            |
| BHD6       | Male   | 65         | >5         | Bilateral Nx | PTX, PC       | NED (25)                            |
| BHD7       | Male   | 56         | 1          | Lt. Nx     | PTX, PC       | Lung metastasis                    |
| BHD8       | Male   | 79         | 3          | Unoperated | PTX, PC, Prostatic Ca. | DOD (78) |
| BHD9       | Female | 68         | 3          | Lt. NSS    | PTX, PC       | NED (18)                            |
| BHD10      | Female | 45         | 5          | Lt. Nx, | PTX, PC       | AWD (390)                           |
| BHD11      | Male   | 48         | 2          | Lt. Nx, | Thyroid Ca.   |                                      |
|            |        |            |            | Rt. NSS    | PTX, PC       | NED (174)                           |
| BHD12      | Female | 29         | 1          | Lt. NSS    | PTX, PC       | NED (19)                            |
| BHD13      | Male   | 63         | 2          | Rt. Nx     | PTX, PC       | NED (18)                            |
| BHD14      | Female | 46         | 1          | Rt. NSS    | PC            | NED (9)                             |
| BHD15      | Female | 65         | 1          | Lt. NSS    | PTX, PC       | NED (9)                             |
| BHD16      | Male   | 45         | 3          | Lt. Nx     | PTX, PC       | NED (9)                             |
| BHD17      | Female | 49         | 2          | Lt. and Rt. NSS | PC            | NED (9)                             |

AWD, alive with disease; Ca, carcinoma; DEAD, died of prostatic cancer; DOD, died of disease; Lt., left; NED, no evidence of disease; NSS, nephron-sparing surgery; Nx, nephrectomy; PC, pulmonary cysts; PTX, pneumotheorax; RCC, renal cell carcinoma; Rt., right; VATS, video-associated thoracic surgery.
Biosystems). done using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Cleveland, OH, USA) and DNA sequencing was obtained using RNeasy Mini kit (Qiagen) according to the manufacturer's instructions.

The PCR conditions were described in our previous study.(13) After purification, DNA was labeled with the Big T13S1ager SYBR Green PCR kit for Quantitative RT-PCR. Conditions for PCR were as follows: 50°C for 2 min, 95°C for 15 min, 40 cycles at 95°C for 30 s, and 60°C for 30 s. GAPDH was used as an internal control gene. The primers used for real-time PCR were: GPMB, (F) 5'-TGCGAGATCAAACGAC-ACA-3' and (R) 5'-CGTGAGCA-CCATTGAAGGT-3'. GAPDH, (F) 5'-CCACCCATG GCAAATTCC-3' and (R) 5'-TGATGGGATTTCCATGAGC-3'. The mRNA levels were expressed as the absolute number of copies normalized against GAPDH mRNA. Differences in amplification were determined using the standard curve method.

Somatic mutation analysis of FLCN in renal tumors. DNA was extracted from renal tumor tissues. If only the germline mutational signal was amplified and the wild-type sequence was unreadable, it was determined that LOH occurred as the second hit. Exons other than genetically mutated sites were also amplified, and if somatic intragenic mutation was suggested, the PCR product was subcloned and sequenced to clarify the second hit.

Results

Clinical histories and FLCN mutation patterns. Patient characteristics are summarized in Table 1. All the patients in this study were Japanese, and the patient group included nine women and nine men. Two patients had lung metastases. One of them (BHD7) had a 10-cm-sized solitary RCC and multiple lung metastases at the time of diagnosis. The patient died...
78 months after nephrectomy and successive chemotherapy and targeting therapies. All of the patients had pulmonary cysts and four patients had skin tumors.

The C8 tract in exon 11 (c.1285dupC) was the most frequent germline mutation pattern. It was detected in 9 of 18 patients. Deletion of GATG in exon 13 (c.1533_1536delGATG) was detected in four patients. Other affected families showed different mutation patterns (Table 2).

In the sporadic RCC group, one patient with unilateral chromophobe RCC had a few small air cystic spaces in the lung. Although the patient and family members had neither fibrofolliculoma nor pneumothorax, genetic testing was carried out. No mutation was detected, and the possibility of BHD was excluded. Other patients diagnosed with sporadic tumors did not have any manifestation related to BHD, thus genetic testing was not carried out.

**Histology of FLCN-related renal tumors.** Two patients were excluded from histological study. Patient BHD8 died of a prostatic tumor and had not undergone surgery; at autopsy, renal tumors showed autolysis. Patient BHD6 experienced chronic renal failure. Twelve tumor lesions were pathologically diagnosed as chromophobe RCCs (44.4%), six tumors were HOCTs (22.2%), three tumor lesions were papillary RCCs (11.1%), and two tumor lesions were clear cell RCCs (7.4%). In BHD4, one of three tumors (BHD4-T3) was composed of more than one histological type. In BHD16, two tumors were mostly replaced by calcified material. In BHD2-proband, two independent tumors were diagnosed as unclassified.

Chromophobe RCCs and HOCTs grew in an expansive manner. A few cystic tubules were composed of low columnar benign-looking cells that were intermingled in the peripheries or the centers of the tumors. That is, intratumoral peripheral small papillary tufts were associated with fibrotic stroma or thick-walled blood vessels (Fig. 1a,b). In some areas, tumor cells looked clear due to intracytoplasmic vacuoles (Fig. 1c,d).
Somatic mutations of \textit{FLCN}. Possible somatic mutations of \textit{FLCN} were investigated in 16 tumors (7 freshly frozen tumors and 9 paraffin-embedded tumors). Second hit intragenic mutations were detected in 6 tumors. In these tumors, the second hits occurred in a different exon from the genetically affected exon (Table 2). Among them, in BHD9-T2, monoallelic mutations between exon 10 and exon 11 were identified by the cloning (Figs 2a, S1). It was unknown in the other five tumors which allele was affected due to large-sized intervening introns or the quality of DNA from paraffin-embedded tumors. Apart from six tumors with second hits, two tumors showed LOH (Fig. 2b) in which the heterozygous mutation pattern was completely replaced by a hemizygous mutation pattern. The germ-line mutation was retained and the wild-type allele was lost, thus both copies were affected.

Expression of FLCN protein in BHD and sporadic renal tumors. Next, we investigated FLCN protein expression. Using the antibody D14G9 for Western blotting, the FLCN bands (70 kDa) stained strongly in non-tumor regions of BHD kidneys, whereas FLCN bands were either undetectable or much weaker in RCCs of BHD patients, compared with the corresponding non-tumor parts (Fig. 3a). In sporadic tumors, the FLCN bands were clearly detected (Fig. 3b). We also obtained similar results using antibodies sc-25168 and ab93196 (data not shown).

We carried out FLCN immunostaining using the antibody ab93196 (Fig. 4). The staining sensitivity and intensity of this
Table 3. Summary of folliculin (FLCN) and glycoprotein non-metastatic B (GPNMB) immunostaining in Birt–Hogg–Dubé (BHD) and sporadic renal tumors

| Immunostaining | HOCT/oncocytoma | Chromophobe | Papillary | Clear cell |
|----------------|-----------------|-------------|-----------|------------|
|                | BHD, n = 6 (%)  | Sporadic, n = 8 (%) | BHD, n = 9 (%) | Sporadic, n = 18 (%) | BHD, n = 2 (%) | Sporadic, n = 11 (%) | BHD, n = 2 (%) | Sporadic, n = 20 (%) |
| FLCN Nuclear    | 0 (0)           | 6 (75)      | 2 (22)    | 11 (61)    | 0 (0)           | 9 (82)      | 0 (0)           | 16 (80)     |
| Cytoplasmic     | 5 (83)          | 2 (25)      | 7 (78)    | 6 (33)    | 1 (50)          | 0 (0)       | 0 (0)           | 1 (5)       |
| Negative        | 1 (17)          | 0 (0)       | 0 (0)     | 1 (6)    | 1 (50)          | 2 (18)      | 2 (100)         | 3 (15)      |
| GPNMB (-)       | 0 (0)           | 8 (100)     | 0 (0)     | 9 (50)    | 1 (50)          | 10 (91)     | 2 (100)         | 20 (100)    |
| (+)             | 1 (17)          | 0 (0)       | 2 (22)    | 8 (44)    | 0 (0)           | 1 (9)       | 0 (0)           | 0 (0)       |
| (+++)           | 5 (83)          | 0 (0)       | 7 (78)    | 1 (6)    | 1 (50)          | 0 (0)       | 0 (0)           | 0 (0)       |

(-), No staining or <5% of the tumor cells positive; (+), occasional weak staining; (++), moderate or intense staining. HOCT, hybrid oncocytic chromophobe tumor.

Fig. 4. Folliculin (FLCN) immunostaining in normal kidney, and Birt–Hogg–Dubé (BHD) and sporadic tumors. (a) H&E staining of a normal kidney. (b) Serial section of sample A immunostained for FLCN. Nuclei of distal tubules were strongly positive for FLCN. (c–h) Immunostaining for FLCN in BHD and sporadic tumors. Weak cytoplasmic staining was evident in tumors of BHD patients (c, e, h). Insets highlight distal tubules of non-tumor areas, showing immunoreactivity to FLCN. Sporadic renal tumors (d, f, h) showed strong nuclear staining.
product were the best among the three examined antibodies. In normal renal cortices, FLCN was strongly stained in the nuclei and cytoplasms of distal tubules and collecting ducts (Fig. 4a,b).

The majority of FLCN-related tumors showed weak cytoplasmic staining (Table 2, Fig. 4c,e,g). Nuclear staining was observed in only two FLCN-related tumors. In normal areas of BHD kidneys, strong nuclear staining was detected (Fig. 4c,e,g, insets). In sporadic cases, most tumors (except chromophobe RCCs) showed nuclear staining. In chromophobe RCCs, 11 of 18 cases showed nuclear staining (61.1%) (Table 3, Fig. 4d,f,h). Overexpression of GPNMB protein in FLCN-related tumors.

Western blotting clearly indicated the loss of FLCN, but immunostaining for FLCN was not sufficiently specific to predict RCC associated with BHD. Some auxiliary markers were desirable to give an indication for genetic analysis. In the current study, we compared GPNMB expression between BHD and sporadic tumors. In quantitative RT-PCR, RCCs from BHD patients expressed GPNMB mRNA at levels that averaged 23-fold higher compared to sporadic RCCs (P < 0.01, Student’s t-test) (Fig. 5a). GPNMB mRNA levels of non-neoplastic renal tissues of BHD patients were as low as those of sporadic RCCs (data not shown).

In Western blot analysis, strong expression of GPNMB protein was detected only in FLCN-related tumors, and the bands were undetectable in non-tumor renal tissues of BHD patients. The GPNMB bands were barely detectable in sporadic RCCs and normal kidneys (Fig. 5b,c).

Immunostaining revealed that GPNMB was undetectable in normal control kidneys (Fig. 6). All FLCN-related RCCs (except for three non-chromophobe RCCs) were positively stained for GPNMB (Table 2, Fig. 6b,d). Immunostaining intensities of GPNMB were correlated with the relative expression of GPNMB mRNA, such that BHD15 with intense staining showed the highest expression whereas BHD2 with moderate staining showed lower expression compared with BHD15. Small nodules of renal oncocytosis in non-tumor regions, which is one of the diagnostic characteristics of BHD kidneys, also showed moderate immunostaining (Fig. 6f). In sporadic cases, half of the chromophobe RCCs and most other types were negatively stained for GPNMB (Table 3). Importantly, all sporadic oncocytomas were negative for GPNMB, whereas all HOCTs in BHD were positively stained. With regard to GPNMB-positive RCCs, all but one showed nuclear staining for FLCN. Only one GPNMB-positive RCC showed cytoplasmic staining for FLCN. The patient with this tumor also had a few pulmonary cysts. Genetic testing was carried out and the possibility of BHD was eliminated.

Discussion

Several important signaling pathways are affected by FLCN deficiency, including mTOR and the MiTF family. (12,16–18) We confirmed overexpression of phospho-S6, a downstream molecule of mTOR complex 1, in FLCN-related RCCs (data not shown). However, mTOR pathway molecules are also expressed in sporadic RCCs. (19,20) These ubiquitously upregulated molecules cannot be used for differential diagnosis.

A previous analysis of FLCN-related RCCs revealed frequent somatic frameshift mutations or LOH. (21) In situ hybridization analysis indicated that FLCN mRNA was undetectable in the RCCs of BHD patients. (22) The results of the current study supported the notion that the intact form of FLCN protein is lost in RCCs of BHD patients. However, immunohistochemical studies of a substantial number of FLCN-related RCCs have not been carried out due to the specificity and sensitivity of antibodies. (23) Investigators using a non-commercial polyclonal antibody reported strong immunostaining for FLCN in a BHD renal tumor with a second hit in the FLCN gene. (24) We recognize that the antibody ab93196 showed strong nuclear staining in normal renal tubules. HeLa cells transiently transfected with FLCN showed nuclear staining in vitro. (16) Nuclear accumulation of FLCN in normal cells has also been described in published reports. (23) Therefore, a weak cytoplasmic staining in vivo might alert us to the possibility of BHD. The pathophysiological significance of nuclear localization of FLCN is poorly understood, and further study is needed.

In the present study, the expression of GPNMB mRNA was significantly higher in FLCN-related RCCs than in sporadic cases, indicating that GPNMB is a useful marker for FLCN-related tumors.
tumors, data that were supported by Western blotting. However, half of the sporadic chromophobe RCCs showed weak immunostaining for GPNMB. It should be noted that all of these RCCs showed a nuclear staining for FLCN. Although immunoreactivity for GPNMB alone cannot be sufficiently specific to predict RCC associated with BHD, a combined immunohistochemical panel for FLCN and GPNMB might predict appropriate cases for genetic testing. One sporadic RCC case with pulmonary cysts showed the immunostaining patterns suggestive of FLCN-related tumors. Although genetic testing excluded the possibility of BHD, careful follow-up is needed to better understand the unusual clinicopathological features of this case. Sporadic chromophobe RCCs rarely have somatic FLCN mutations but potentially have promoter methylation and LOH. Therefore, further studies are needed to understand the role of FLCN in sporadic RCCs.

Little is known about the roles of GPNMB in the human kidney. Glycoprotein non-metastatic B is upregulated in monocytes and macrophages in dialysis patients, suggesting that it has a role as an inflammatory mediator in a uremic milieu. Glycoprotein non-metastatic B plays an essential role in osteoblast maturation and osteoclast differentiation and is also involved in melanosome formation. In previous work on GPNMB in FLCN signaling, FLCN inactivation induced nuclear accumulation of TFE3, allowing the production of GPNMB. We also examined immunostaining of TFE3 in the current cases, but TFE3-positive cells were sparse (data not shown), suggesting that the overexpression of GPNMB might not be an event explained solely by the FLCN–TFE3 axis. Not only MiTF family members but also growth factors were reported to be involved in the cascade. For example, β-fibroblast growth factor and platelet-derived growth factor were shown to induce GPNMB overexpression in skeletal muscle cells. Although the detailed role of GPNMB in RCC needs to be analyzed in greater detail, the present study suggests that GPNMB is expressed more abundantly in FLCN-related RCC than sporadic RCC.

We suggest that urologists and pathologists should carefully analyze the background of unusual renal tumors with multifocal growth. It is hoped that our study of FLCN and GPNMB expression might help distinguish FLCN-related RCCs from sporadic tumors. It is important to understand the detailed role of GPNMB in renal carcinogenesis in BHD patients.
studies will also be needed to find additional useful markers in the diagnosis of FLCN-related RCCs.

Acknowledgments

The authors thank Drs. Hiroko Gotoda, Yasushi Yatabe, Akira Okada, Suzuko Moritani, Fumi Kawakami, Toshiharu Sakuma, Shin Sasaki, Tomohiro Hasaka, Yoshiaki Imamura, Muneyuki Hiraishi, Yoshinori Matsui, and Keisuke Sugimoto for providing clinical information and renal tissues, and members of pathology laboratories in Yokohama City University Graduate School of Medicine and affiliated hospitals for excellent assistance, and Ms. Y Imanishi for sequence analysis. This work was supported by Japan Society for the Promotion of Science (Grant Nos. 23590406 [to M.F.] and 24590408 [to Y.N.]), the Ministry of Health, Labor and Welfare (Y.N.), Yokohama Foundation for Advancement of Medical Science (M.F.), Suzuki Hinyoki Igaku Shinko Zaidan (M.F.) and the Mitsubishi Foundation (M.F.).

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Cloning from exon 10 to exon 11 of folliculin (FLCN) in Birt–Hogg–Dube (BHD) syndrome patient BHD9-T2. Exon 10 (114 bp) and exon 11 (124 bp) are intervened by intron 10 (565 bp). The sequence results of four clones (Nos. 1C–4C) are shown. Ex10 and Ex11 indicate wild-type sequences. Clone-2 (No. 2C) simultaneously shows somatic mutation in exon 10 (second line from the top) and germline mutation in exon 11 (bottom line).

Disclosure Statement

The authors have no conflict of interest.

Abbreviations

BHD Birt–Hogg–Dube syndrome
FLCN folliculin
GPMB glycoprotein non-metastatic B
HOCT hybrid oncocytic/chromophobe tumor
LOH loss of heterozygosity
mTOR mammalian target of rapamycin
RCC renal cell carcinoma
TFE3 transcription factor E3

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Furuya et al.