

## **The Inaugural Birt-Hogg-Dubé (BHD) Symposium**

**Roskilde, Denmark, September 3rd, 2008**

### **Scientific Organising Committee**

Professor Eamonn Maher (chair)

Dr Fred Menko

Dr Laura Schmidt

Dr Jorge Toro

Dr Maurice van Steensel

## Programme

Morning	Molecular	Chair	Time	Oral Presentation Title	Speaker
9:35	Registration 5 min intro				
09:40–10:55	5 * 15 min presentations	Laura Schmidt	9:40	Inactivation of the BHD gene in mice results in early embryonic lethality: the expression pattern and role of BHD in mouse embryo development	Yukiko Hasumi
			9:55	Role of the Birt-Hogg-Dubé Tumor Suppressor in Embryonic Development	Tim Cash
			10:10	Functional Characterization of the BHD Tumor Suppressor Protein	Arnim Pause
			10:25	FNIP1 inactivation in vivo leads to B-cell developmental defects: phenotypic analysis of a FNIP1 knockout mouse model	Masaya Baba
			10:40	Identification and characterization of a novel folliculin-interacting protein FNIP2	Hisashi Hasumi
10:55–11:25	Coffee				
11:25–12:00	LS keynote molecular presentation	Jorge Toro			
12:00–12:45	3 * 15 min presentations	Jorge Toro	12:00	Interaction of folliculin (Birt-Hogg-Dube gene product) with a novel Fnip1-Like (FnipL) protein	Yumiko Takagi
			12:15	Restoration of FLCN suppresses tumorigenic potential of a BHD patient-derived renal cancer cell line: Identification of the genes and signaling pathways affected by FLCN expression	Laura Schmidt
			12:30	Folliculin-dependent regulation of HIF2-alpha	Sherry Wepler
12:45–14:10	Lunch (posters and EBC meeting)				
Afternoon	Clinical	Chair	Time	Oral Presentation Title	Speaker
14:10–14:45	ML keynote clinical presentation	Maurice van Steensel			
14:45–15:45	4 * 15 min presentations	Maurice van Steensel	14:45	Mutation analysis of the Birt-Hogg-Dubé gene in patients with multiple lung cysts	Makiko Kunogi
			15:00	Analysis of the BHD gene in a series of European patients suspected of Birt-Hogg-Dubé syndrome	Sophie Giraud
			15:15	Dermatologic Investigations of Birt-Hogg-Dubé Syndrome at the National Cancer Institute	Jorge Toro
			15:30	High-Resolution CT findings of the chest in 12 patients of Birt-Hogg-Dube syndrome.	Kazunori Tobino
15:45–16:15	Coffee				
16:15–17:30	Patient data and treatment	Fred Menko	16:15	EBC patient data	Eamonn Maher
			16:30	Provisional Diagnostic Criteria, Genetic Testing and Recommendations for Screening and Surveillance Patients with Birt-Hogg-Dubé Syndrome	Jorge Toro
			16:45	Panel discussion—treatment, future etc.	

## Abstracts

### Oral Presentations

001

#### Inactivation of the BHD gene in mice results in early embryonic lethality: the expression pattern and role of BHD in mouse embryo development

Yukiko Hasumi<sup>1</sup>, Rieko Ajima<sup>2</sup>, Masaya Baba<sup>1</sup>, Serguei V. Kozlov<sup>3</sup>, Diana C. Haines<sup>4</sup>, Hisashi Hasumi<sup>1</sup>, Seung-Beom Hong<sup>1</sup>, Terry P. Yamaguchi<sup>2</sup>, W. Marston Linehan<sup>1</sup>, Laura S. Schmidt<sup>1,5</sup>

<sup>1</sup>Urologic Oncology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, 20892, USA; <sup>2</sup>Cell Signaling in Vertebrate Development Section Cancer and Developmental Biology Laboratory, National Cancer Institute-Frederick, Frederick, Maryland, 21702, USA; <sup>3</sup>Center for Advanced Preclinical Research; <sup>4</sup>Pathology/Histotechnology Laboratory; <sup>5</sup>Basic Research Program, SAIC-Frederick, Inc., National Cancer Institute-Frederick, Frederick, Maryland, 21702, USA.  
Tel: +1-301-451-8141, Fax: +1-301-402-0922,  
e-mail: hasumiy@mail.nih.gov

**Background:** Birt-Hogg-Dubé syndrome, a hamartoma disorder characterized by benign tumors of the hair follicle, lung cysts, and renal neoplasia, is caused by germ-line mutations in the BHD (FLCN) gene, which encodes a tumor-suppressor protein, folliculin (FLCN), with unknown function.

**Method:** To examine the role of BHD in vivo, we established mice with BHD null (delete, d) and BHD loxP-flanked (flox, f) conditional knockout alleles.

**Results:** Virtually all BHD null (d/d) mutants in a mixed (129 Sv × C57BL/6) genetic background died before E6.5. Compared to the BHD wild-type or heterozygous null embryos, BHD d/d embryos at E6.5 exhibited a smaller size with an irregular surface. In addition, the effects of acute inactivation of BHD during the intermediate stage of mouse embryogenesis were analyzed in BHD f/d, CreERTM mice in which BHD was deleted by Cre recombinase in a tamoxifen-inducible manner. Using whole mount in situ hybridization techniques, we found that BHD mRNA was ubiquitously expressed even in early stages of mouse development.

**Conclusion:** These results suggest an essential requirement for BHD in embryonic development and survival  
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002

#### Role of the Birt-Hogg-Dubé tumor suppressor in embryonic development

Cash Timothy P., Simon M. Celeste

Abramson Family Cancer Institute, University of Pennsylvania, Philadelphia, PA, USA

To assess the role of the Birt-Hogg-Dubé (BHD) gene in embryonic development, we have utilized a gene-trap mouse model, kindly provided by Dr. Elizabeth Henske at Fox Chase Cancer Center. This mouse model bears a gene-trap cassette in intron 8 of the murine Bhd locus, which effectively ablates transcription downstream of exon 7. Genotypic analysis of embryos from Bhd +/- intercrosses demonstrates that Bhd -/- embryos are lethal before e7.5, with Bhd -/- e6.5 egg cylinders showing severe gross defects. In order to understand the molecular nature of these defects, we have created two independent Bhd embryonic stem (ES) cell lines in vitro, each bearing

a unique mutant Bhd allele. Consistent with previous findings<sup>1,2</sup>, we have observed increased Akt-mTOR and ERK activation in Bhd -/- ES cells. This phenomenon appears specific to ES cells, since transient knockdown of BHD in differentiated cell types does not yield a similar effect. From the ES cells, we have also generated embryoid bodies to study the role of BHD in primary germ layer formation and hematopoietic stem cell (HSC) development and have observed that Bhd -/- ES cells fail to form HSC lineages in methylcellulose cultures. Taking advantage of a  $\beta$ -galactosidase reporter in the gene trap cassette, we have also observed that Bhd is expressed in embryonic HSC compartments in vivo, further suggesting a novel role for BHD in HSC maintenance and/or differentiation.

1. Baba M, Hong SB, Sharma N, Warren MB, Nickerson ML, Iwamatsu A, Esposito D, Gillette WK, Hopkins RF 3rd, Hartley JL, Furihata M, Oishi S, Zhen W, Burke TR Jr, Linehan WM, Schmidt LS, Zbar B. 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 U S A. 2006 Oct 17;103(42): 15552-7.

2. Baba M, Furihata M, Hong SB, Tessarollo L, Haines DC, Southon E, Patel V, Igarashi P, Alvord WG, Leighty R, Yao M, Bernardo M, Ileva L, Choyke P, Warren MB, Zbar B, Linehan WM, Schmidt LS. 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 Jan 16;100(2): 140-54.

Contact information:

Timothy Cash

E-mail: tycash@mail.med.upenn.edu

Tel.: +01 215-746-5526

Dr. M. Celeste Simon, Ph.D.

E-mail: celeste2@mail.med.upenn.edu

Tel.: +01 215-746-5526

003

#### Functional characterization of the BHD tumor suppressor protein

Valérie Hudon, Vasiliki Kottis, Noriko Uetani, Arnim Pause

McGill Cancer Center and Department of Biochemistry, McGill University, McIntyre Building, Room 716, 3655 Sir William Osler Promenade, Montreal (Québec), H3G 1Y6, Canada  
Tel: 514-398-1557

Fax: 514-398-6769

E-mails: valerie.hudon@mail.mcgill.ca; vicky.kottis@mcgill.ca; noriko.uetani@mcgill.ca; arnim.pause@mcgill.ca

The Birt-Hogg-Dubé (BHD) syndrome is a hereditary human cancer syndrome characterized by a predisposition to develop renal carcinoma, pulmonary cysts, colorectal and endometrial tumors. Affected family members present germline mutations in a novel gene, BHD or FLCN. The functions of the protein encoded by FLCN, folliculin, are still unclear. In the present study, we developed a Flcn knockout mouse model using gene-trapping technology to characterize the functions of folliculin in tumor formation.

We found that homozygous Flcn knockout mice died in utero at an early stage of fetal life. We characterized the pattern of expression of folliculin in the adult mouse and observed that folliculin is expressed in most tissues of the adult mouse including kidneys, lungs, brain, cerebellum and heart. Our mouse model developed early renal lesions such as tubular hyperplasia with or without cysts as well as rare papillary adenoma however no carcinoma was observed so far. Since our mouse model manifests early preneoplastic kidney lesions that seems to progress toward malignancy, we will characterize the underlying pathways for the initiation of tumorigenesis and progression.

In addition, we show tumor suppressor activity of FLCN in a mouse xenograft assay. To this end, we used folliculin negative RCC

cells and generated stable cell lines with restored expression of FLCN. We also produced stable FLCN knockdown in a RCC cell line, which leads to increase tumor formation in the xenograft assay. Currently, we are characterizing the signaling pathways regulated by FLCN in these tumors that are linked to growth control.

#### 004

##### **FNIP1 inactivation in vivo leads to B-cell developmental defects: phenotypic analysis of a FNIP1 knockout mouse model**

Masaya Baba<sup>1</sup>, Hyung-Chan Suh<sup>2,3</sup>, Jonathan R. Keller<sup>2,3</sup>, Seung-Beom Hong<sup>1</sup>, Hisashi Hasumi<sup>1</sup>, Yukiko Hasumi<sup>1</sup>, W. Marston Linehan<sup>1</sup>, Laura S. Schmidt<sup>1,3</sup>

<sup>1</sup>Urologic Oncology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, 20894, United States, Phone: 301-496-6353; fax: 301-402-0922;

<sup>2</sup>Center for Cancer Research, National Cancer Institute-Frederick, Frederick, MD 21702, United States, Phone: 301-846-1461; Fax: 301-846-6646; <sup>3</sup>Basic Research Program, SAIC-Frederick, Inc., National Cancer Institute-Frederick, Frederick, Maryland 21702, United States

Bioinformatic analysis of the BHD protein, folliculin (FLCN) revealed no known functional domains. In order to elucidate FLCN function, we previously identified a novel FLCN binding protein FNIP1 that interacts with 5'-AMP activated protein kinase. To investigate FNIP1 function in vivo, we have generated a FNIP1 knockout mouse model. A gene trap cassette was inserted into intron 2 of mouse FNIP1, thereby disrupting normal transcription of the FNIP1 gene. We found no obvious phenotypic differences between wild type and FNIP1 heterozygous knockout mice. FNIP1 homozygous knockout mice were viable, but developed spleens with reduced size. We analyzed bone marrow cells and splenocytes by FACS analysis and found that FNIP1 homozygous knockout mice lack mature B-cells. B-cell development was blocked at the pro-B to pre-B cell transition stage. To evaluate the ability of pro-B cells to differentiate to pre-B cells, we co-cultured pro-B cells with OP9 stromal cells in vitro. Although heterozygous FNIP1 knockout pro-B cells were able to differentiate, homozygous FNIP1 knockout pro-B to pre-B cell transition was arrested confirming a cell autonomous defect in B-cell differentiation. We will discuss potential molecular mechanisms to explain the B-cell developmental defects in FNIP1 homozygous knockout mice.

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E-mails:

MB: babam@mail.nih.gov

HS: hcsuh@ncifcrf.gov

JK: kellerj@ncifcrf.gov

SH: shong@ncifcrf.gov

HH: hasumih@mail.nih.gov

YH: hasumiy@mail.nih.gov

WML: linehanm@mail.nih.gov

LS: schmidt@mail.nih.gov

#### 005

##### **Identification and characterization of a novel folliculin-interacting protein FNIP2**

Hisashi Hasumi<sup>a</sup>, Masaya Baba<sup>a</sup>, Seung-Beom Hong<sup>a</sup>, Yukiko Hasumi<sup>a</sup>, Ying Huang<sup>c</sup>, Masahiro Yao<sup>c</sup>, Vladimir A. Valera<sup>a</sup>, W. Marston Linehan<sup>a</sup>, Laura S. Schmidt<sup>a,b</sup>

<sup>a</sup>Urologic Oncology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, 20894, United States; <sup>b</sup>Basic Research Program, SAIC-Frederick, Inc., National Cancer Institute-Frederick, Frederick, Maryland 21702, United States; <sup>c</sup>Department of Urology and Molecular Genetics, Yokohama City University Graduate School of Medicine, Yokohama, Japan  
E-mail: hasumih@mail.nih.gov (Hisashi Hasumi)  
Tel: +1 301 451 8143

Birt-Hogg-Dubé syndrome characterized by increased risk for renal neoplasia is caused by germline mutations in the BHD/FLCN gene encoding a novel tumor suppressor protein, folliculin (FLCN), which interacts with FNIP1 and 5'-AMP-activated protein kinase (AMPK). Here we report the identification and characterization of a novel FNIP1 homolog FNIP2 that also interacts with FLCN and AMPK. C-terminally deleted FLCN mutants, similar to those produced by naturally-occurring germline mutations in BHD patients, were unable to bind FNIP2. These data taken together with our previous results that demonstrated FNIP1 binding to the C-terminus of FLCN suggest that FLCN tumor suppressor function may be facilitated by interactions with both FNIP1 and FNIP2 through its C-terminus. Furthermore, we demonstrate that FNIP1 and FNIP2 are able to form homo- or heteromeric multimers suggesting that they may function independently or cooperatively with FLCN. Differential expression of FNIP1 and FNIP2 transcripts in some normal tissues may indicate tissue specificity for these homologs. Interestingly FNIP1 and FNIP2 were oppositely expressed in human clear cell renal cell carcinoma (RCC), and coordinately expressed in chromophobe RCC and oncocytoma, suggesting their differential function in different histologic variants of RCC.

#### 006

##### **Interaction of folliculin (Birt-Hogg-Dubé gene product) with a novel Fnip1-Like (FnipL) protein**

Y. Takagi<sup>1,2</sup>, T. Kobayashi<sup>1</sup>, K. Takahashi<sup>2</sup>, and O. Hino<sup>1</sup>

Departments of <sup>1</sup>Pathology and Oncology and <sup>2</sup>Respiratory Medicine, Juntendo University School of Medicine, Bunkyo-Ku, Tokyo 113-8421, Japan  
Tel: +81 358021039; Fax: +81 356841646;  
E-mail: yumikogt@med.juntendo.ac.jp

The function of Birt-Hogg-Dubé gene (BHD) gene product, folliculin (Flcn), is totally unknown, although its interaction with Fnip1 and involvement in mTOR pathway have been reported. In this study we identified FnipL, a novel protein binding to Flcn, which is highly homologous to Fnip1. The interaction between FnipL and Flcn may be mediated mainly by the C-terminal domains of each protein as well as Flcn-Fnip1 interaction. FnipL and Flcn were located together in the cytoplasm in a reticular pattern, although solely expressed Flcn was found mainly in the nucleus. Cytoplasmic retention of Flcn was canceled with C-terminal truncation of FnipL, suggesting that FnipL regulates Flcn distribution through their complex formation. FnipL bound and phosphorylated by AMPK. By the employment of siRNA, we observed a decrease in S6K1 phosphorylation in the BHD-suppressed HeLa cell. We also observed a decrease in S6K1 phosphorylation in FNIP1- and, to a lesser extent, in FNIP1-suppressed HeLa cells. These results suggest that, at least in some cell-types or conditions, Flcn-FnipL and Flcn-Fnip1 complexes positively regulate S6K1 phosphorylation, and that FnipL provides an important clue to elucidating the function of Flcn and the pathogenesis of disease.

007

### Restoration of FLCN suppresses tumorigenic potential of a BHD patient-derived renal cancer cell line: Identification of the genes and signaling pathways affected by FLCN expression

Seung-Beom Hong<sup>1</sup>, Jaime Stull<sup>1</sup>, Masaya Baba<sup>1</sup>, Maria J. Merino<sup>2</sup>, W. Marston Linehan<sup>1</sup> and Laura S. Schmidt<sup>1,3</sup>

<sup>1</sup>Urologic Oncology Branch, and <sup>2</sup>Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892; <sup>3</sup>Basic Research Program, SAIC-Frederick, Inc., National Cancer Institute-Frederick, Frederick, MD 21702

Contact Information:

Seung-Beom Hong, E-mail: hongse@mail.nih.gov, Tel: 1-301-594-9547.

Jaime Stull: jstull@Udel.Edu

Masaya Baba: babam@mail.nih.gov, Tel: 1-301-451-8137.

W. Marston Linehan: linehanm@mail.nih.gov, Tel: 1-301-496-6353.

Laura S. Schmidt: schmidt@mail.nih.gov, Tel: 301-402-4707.

Maria J. Merino: mjmerino@box-m.nih.gov, Tel: 301-496-3326.

Germline mutations in the FLCN (BHD) gene are responsible for the development of fibrofolliculomas, lung cysts and renal neoplasia in Birt–Hogg–Dubé syndrome. To examine the tumor suppressor function of FLCN, wild-type or mutant FLCN (H255R) was stably expressed in a renal tumor cell line, UOK257, that harbors a cytosine insertion mutation in FLCN (c.1733insC) with concomitant loss of the wild-type copy of FLCN. The growth rate of the cell lines in culture was not dependent on the expression of wild-type FLCN. However, when the cells were injected into nude mice, tumor development was inversely dependent upon the level of wild-type FLCN expression. The parental UOK257 and mutant FLCN-H255R-expressing UOK257 cell lines formed solid tumors at high frequency with high grade clear cell histology. One of the cell lines, UOK257-3, in which a low level of wild-type FLCN was expressed, generated tumors of smaller size at a lower frequency. The cell lines, UOK257-2, -4 and -6, which expressed high levels of FLCN, produced no solid tumors over a one-year period. To identify the downstream target genes of FLCN, we compared the gene expression patterns of the mutant and wild-type FLCN cell lines using microarray analysis. We identified differentially expressed genes involved in cancer-associated cell signaling pathways including TGF- $\beta$ /BMP, Jak/Stat, integrin, apoptosis, and inflammation signaling, as well as genes important for angiogenesis. Our study confirms the tumor suppressor function of FLCN using an *in vivo* model and identifies new downstream target genes and putative pathways that may contribute to tumor suppression by FLCN.

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008

### Folliculin-dependent regulation of HIF2-alpha

Weppler, S.A., Claessens, T., van Geel, M, and van Steensel, M.A.M.

Maastricht University Medical Centre, Department of Dermatology, PO Box 5800, 6202 AZ Maastricht, The Netherlands  
Email: mauricevansteensel@gmail.com  
Tel: +31 43 387 7292

Birt–Hogg–Dubé (BHD) syndrome results from a germline mutation of the gene encoding folliculin. The majority of documented mutations are predicted to result in truncation of the protein. In order to simulate loss of functional folliculin expression and to investigate the molecular consequences of BHD syndrome, we have established a

stable knock-down model using HEK293 cells in which expression of the BHD gene has been reduced using short-hairpin RNA. We demonstrate a decrease of ~50% in the expression of folliculin mRNA as assessed by quantitative RT-PCR, which is comparable to the expression level found in the tissues of BHD patients. Molecular characterization of the folliculin knock-down cells revealed increased cyclin D and decreased p27 mRNA expression, suggesting that cell proliferation may be influenced by folliculin. However, the growth rate of folliculin knock-down cells was not different from control cells under normal culture conditions. We are currently investigating if folliculin plays a role in cell growth under stress conditions such as serum starvation or hypoxia.

We also investigated the expression of hypoxia inducible factors (HIF1 and HIF2 alpha) in our folliculin knock-down cells. We found a modest increase in HIF1-alpha mRNA expression, but a 2–3 fold increase in HIF2-alpha mRNA expression in the folliculin knock-down cells compared to controls. Under hypoxic conditions, HIFs become stabilized and activate the transcription of certain target genes. We see increased expression of Cited2, a HIF2-specific target gene, under both normoxic and hypoxic conditions which suggest that HIF2 activity is elevated in the folliculin knock-down cells. Reduction in folliculin expression was also associated with increased survival after exposure to hypoxia. These data suggest that BHD patients may have aberrant HIF2 activity which could contribute to the development of both skin and kidney tumours.

**Keywords** HIF2, Hypoxia, HEK293

009

### Mutation analysis of the Birt–Hogg–Dubé gene in patients with multiple lung cysts

Makiko Kunogi<sup>1</sup>, Taeko Akiyoshi<sup>1</sup>, Yoko Gunji<sup>1</sup>, Takako Shigihara<sup>2</sup>, Mika Kikkawa<sup>2</sup>, Noriko Shindo<sup>2</sup>, Toshiyuki Kobayashi<sup>3</sup>, Okio Hino<sup>3</sup>, Kazuhisa Takahashi<sup>1</sup>, Kuniaki Seyama<sup>1</sup>

<sup>1</sup>Department of Respiratory Medicine, Juntendo University School of Medicine; <sup>2</sup>Division of Molecular and Biochemical Research, Biomedical Research Center, Juntendo University Graduate School of Medicine; <sup>3</sup>Department of Pathology and Oncology Juntendo University Graduate School of Medicine  
e-mail: komanogi@med.juntendo.ac.jp  
Phone: +81-3-5802-1063  
Fax: +81-3-5802-1617

**Background:** Birt–Hogg–Dubé (BHD) syndrome, a rare inherited autosomal genodermatosis first recognized in 1977, is characterized by fibrofolliculomas of the skin, an increased risk of renal tumors and multiple lung cysts with spontaneous pneumothorax. Our previous studies and others reported that a BHD germline mutation can be demonstrated in patients who do not have lesions in the skin, kidneys, and lungs.

**Objective:** To analyze a BHD germline mutation in patients with multiple lung cysts of undetermined cause.

**Methods:** We studied 44 patients with multiple lung cysts. Each exon of the BHD gene was amplified using PCR, followed by denaturing high-performance liquid chromatography (DHPLC) and direct sequencing. In patients without any abnormalities detected by DHPLC, we quantified the amount of each exon of the BHD gene in genomic DNA using a real-time quantitative PCR.

**Results:** We found a BHD germline mutation in 25 (56.8%) of the 44 patients. Fifteen unique small nucleotide alterations including 3 nonsense mutations, 7 deletions, 3 insertions and 2 splice site mutations were identified in 23 patients. A genomic deletion of exon 14 or encompassing exons 9 to 14 was identified in 2 patients.

**Conclusion:** Quantification of each exon of the BHD gene by real time PCR is useful and necessary to identify patients with BHD syndrome.

Makiko Kunogi

Department of Respiratory Medicine, Juntendo University School of Medicine, 2-1-1 Hongo Bunkyo-Ku, Tokyo 113-8421, Japan

Phone: +81-3-5802-1063

Fax: +81-3-5802-1617

E-mail: komanogi@med.juntendo.ac.jp

## 010

### Analysis of the BHD gene in a series of European patients suspected of Birt–Hogg–Dubé syndrome

S. Giraud<sup>1</sup>, C. Bourdin<sup>1</sup>, I. Coupier<sup>2</sup>, D. Bessis<sup>3</sup>, A. Toussaint<sup>1</sup>, P. Delobre<sup>1</sup>, P. Berthet<sup>4</sup>, O. Caron<sup>5</sup>, L. Izatt<sup>6</sup>, F. Fellmann<sup>7</sup>, S. Price<sup>8</sup>, M.F. Avril<sup>9</sup>, A. Calender<sup>1</sup>, S. Richard<sup>10</sup> on behalf of the French NCI Network “VHL disease and inherited predisposition to kidney cancer”

<sup>1</sup>Hospices Civils de Lyon, Hôpital E. Herriot, Service de Génétique, France. Tel: 33472117380, Fax: 33472117381, e-mail:

sophie.giraud@chu-lyon.fr; <sup>2</sup>CHU Montpellier, Hôpital Arnaud de Villeneuve, Service de Génétique, France. Tel: 33467330704, Fax: 33467336052; <sup>3</sup>CHU Montpellier, Hôpital St Eloi, Service de Dermatologie, France. Tel: 33467336906, Fax: 33467336958;

<sup>4</sup>Centre F. Baclesse, Onocogénétique, Caen, France. Tel: 33231455165, Fax: 33231455053; <sup>5</sup>Hôpitaux universitaires de Strasbourg, Hôpital Hautepierre, Département d’oncologie, France. Tel: 33388115785, Fax: 33388116360; <sup>6</sup>Guy’s and St Thomas’ Hospital NHS trust, Clinical Genetics, London, United Kingdom. Tel: 02071881364, Fax: 2071881369; <sup>7</sup>CHU Vaudois, Service de Génétique Médicale, Lausanne, Switzerland. Tel: 021/3143376, Fax: 021/3143392; <sup>8</sup>Northampton General Hospital, Department of Clinical Genetics, United Kingdom. Tel: 01604544638, Fax: 01604545988; <sup>9</sup>Service de Dermatologie, Pavillon Tarnier, Hôpital Cochin, AP-HP, Paris, France. Tel: 33158411781, Fax: 33158411675; <sup>10</sup>Centre Pilote Tumeurs Rares AP-HP/INCa, Service d’Urologie, CHU Le Kremlin-Bicêtre et Service de Néphrologie, Hôpital Necker, Paris, France. Tel: 33149596728, Fax: 33149596726

**Background:** germline mutation in BHD gene are associated with predisposition for Birt–Hogg–Dubé syndrome (BHDS), characterized by follicular hamartomas called fibrofolliculomas (FF), lung cysts, pneumothorax (PNO) and various types of renal cell carcinomas (RCC).

**Objective:** to define good indications of BHD analysis in patients with lesions which enter in the spectrum of BHDS.

**Methods:** we reviewed results for analysis in 210 European patients addressed for suspicion of BHDS. Research for point mutation and large rearrangement was performed by sequencing of the coding sequence and multiplex PCR/ liquid chromatography respectively.

**Results:** we identified variants of BHD sequence in 45 patients: 29 frameshifts, 5 nonsense mutations, 6 splice site mutations and 5 missense mutations. There were 27 different mutations; 19 of them have never been described before. We found mutations in more than 80% of patients with association of at least two characteristic lesions. Mutations were identified in 70%, 43% and 4% of patients with isolated lesions: FF or PNO or RCC respectively. We began familial genetic testing and identified 40 additional mutation carriers.

**Conclusion:** association of specific lesions affecting skin and/or lung and/or kidney in a patient or in a family is a good indication of BHD analysis; it is the same for patients with history of multiples fibrofolliculomas or pneumothorax unlike renal tumors. For these last

patients, restricted criteria as early age of onset or specific histology (hybrid oncocyctic-chromophobe tumors, chromophobe RCC and oncocytomas) should be used for analysis.

## 011

### Dermatologic investigations of Birt–Hogg–Dubé syndrome at the National Cancer Institute

Ousmane Toure, Gladys M. Glenn, Ming-Hui, Marston Linehan, Maria Turner, Jorge R. Toro

National Cancer Institute, Bethesda, MD, USA

Birt–Hogg–Dubé syndrome (BHDS) (OMIM 135150) is an autosomal dominant predisposition to the development of cutaneous hamartomas, lung cysts, spontaneous pneumothorax, and kidney neoplasms. Germline mutations in *FLCN* (also known as BHD) are associated with the susceptibility for BHDS. We previously reported 102 families with *FLCN* germline mutations. Ninety per cent of families with BHDS had individuals with multiple 1–5 mm white or skin coloured papules distributed over the face, neck and/or upper trunk and a histologically confirmed fibrofolliculoma (FF). Approximately, 60% of BHDS families with affected family members with a histologically confirmed fibrofolliculoma (FF) also had individuals with a second cutaneous hamartoma. In 10 % of families (10%) the clinical diagnosis of FF was not confirmed histologically. However, 90% these families had at least one individual with a histologically proven trichodiscoma/angiofibroma and/or perifollicular fibroma. Other cutaneous tumors histologically confirmed in individuals with a germline mutation included: basal cell carcinoma, connective tissue nevus, sebaceous hyperplasia, squamous cell carcinoma and malignant melanoma, leiomyoma, dermatofibrosarcoma protuberans, and leiomyosarcoma. Genotyping of normal and tumor tissue pairs did not show LOH in a collection of 30 fibrofolliculomas. New finding in LOH studies in a variety of cutaneous tumors from patients with germline mutations in *FLCN* will be presented.

## 012

### High-Resolution CT findings of the chest in 12 patients of Birt–Hogg–Dubé syndrome

Kazunori Tobino<sup>1</sup>, Kuniaki Seyama<sup>1</sup>, Toyohiro Hirai<sup>3</sup>, Yoko Gunji<sup>1</sup>, Masatoshi Kurihara<sup>2</sup>, Taeko Akiyoshi<sup>1</sup>, Kenogo Koike<sup>1</sup>, Yuza Kodama<sup>1</sup>, Kazuhisa Takahashi<sup>1</sup>

<sup>1</sup>Departments of Respiratory Medicine, Juntendo University, School of Medicine, Japan; <sup>2</sup>Pneumothorax Center, Nissann Koseikai Tamagawa Hospital, Japan, and <sup>3</sup>Departments of Respiratory Medicine, Kyoto University, School of Medicine, Japan

Kazunori Tobino: e-mail tobino@med.juntendo.ac.jp, Phone +81 338133111, Fax: +81 661675867; Kuniaki Seyama: kseyama@med.juntendo.ac.jp, +81 338133111; Toyohiro Hirai: t\_hirai@kuhp.kyoto-u.ac.jp, +81 757513830, Fax: +81 757514643; Yoko Gunji: gunji@med.juntendo.ac.jp, +81 338133111; Masatoshi Kurihara: kuri@tf6.so-net.ne.jp, +81 337001151, Fax: +81 337002090; Kengo Koike: koiken@med.juntendo.ac.jp, +81 338133111; Yuza Kodama: ykodama@med.juntendo.ac.jp, +81 338133111; Kazuhisa Takahashi: kztakaha@med.juntendo.ac.jp, +81 338133111

**Objective:** The purpose of this study was to describe high-resolution CT (HRCT) findings of the chest in Birt–Hogg–Dubé (BHD) syndrome.

**Materials and Methods:** HRCT images of 12 patients with BHD syndrome (3 males and 9 females, mean age 39.9 years) were retrospectively reviewed by two independent observers. The CT scans

were assessed for the presence of pulmonary cyst and other findings. Then the number of cyst, the size (length of long axis), location, and shape (round, oval and irregular) of the individual cysts were analyzed. If any findings other than pulmonary cysts were identified, the characteristics of those findings were recorded in detail.

**Results:** Multiple pulmonary cysts were seen in all patients. Band-like shadows were seen in nine patients, and 8 of these 9 patients had one or more histories of operation for pneumothorax. The total number of cysts was  $132 \pm 111.6$  (mean  $\pm$  sd) and the average size was  $7.9 \pm 2.0$  mm (mean  $\pm$  sd, range from 1.0 to 68.2 mm). The majority of cysts were irregularly shaped ( $76.6 \pm 6.2\%$ ; mean  $\pm$  sd) and about 40% of cysts bordered pleura ( $40.5 \pm 12.3\%$ ; mean  $\pm$  sd). In all patients, lower medial lung zone was most affected by cysts, and cysts next to or including the proximal portion of lower pulmonary veins or arteries were identified

**Conclusion:** Various sizes of multiple irregularly shaped cysts predominantly distributed in lower medial lung zone are characteristic HRCT findings of BHD syndrome.

From

Kazunori Tobino

Departments of Respiratory Medicine, Juntendo University,  
School of Medicine, Japan

E-mail: tobino@med.juntendo.ac.jp

Phone +81 338133111

Fax: +81 661675867

## 1013

### Provisional diagnostic criteria, genetic testing and recommendations for screening and surveillance patients with Birt–Hogg–Dubé syndrome

Jorge R. Toro and Gladys M. Glenn

National Cancer Institute, Bethesda, MD 20852

Provisional clinical diagnostic criteria for Birt–Hogg–Dubé syndrome (BHDS) have been developed based on review of the medical literature, the clinical evaluations of patients and medical consultations worldwide since 1997, and new understandings gained from molecular genetic testing. The provisional criteria will be presented, discussed and evaluated with participants at the Inaugural BHDS symposium. The criteria integrate clinical, histological and radiological findings obtained at dermatologic, pulmonary and renal tumor evaluations, and medical and family histories of patients at risk. It takes into consideration other skin lesions including angiofibromas/trichodiscomas and/or perifollicular fibromas as well individuals with familial spontaneous pneumothorax without dermatologic history. Biological relatives of a diagnosed family member with BHDS should be offered genetic counseling and testing. In BHDS-affected families with an identified BHD germline mutation, biologic relatives should be considered at-risk until proven mutation negative. The mutation status is determined for family members and those with a germline BHD mutation are advised to undergo genetic counseling and clinical screening examinations. Genetic testing eliminates the burden of and unnecessary screening and radiation exposure of individuals not at risk. The provisional screening and surveillance guidelines for individuals at risk for BHDS that will be discussed addresses the aggressiveness of BHDS-associated renal tumors, who is at-risk and should be screened, what imaging modalities should be used, how frequently individuals should be screened, and when to refer to a urologic oncologist. These criteria, screening and surveillance guidelines continue to be modified as more is learned about BHDS and diagnostic genetic testing becomes more widely used.

## Posters

### 101

#### Characterization of germ-line mutations in the *Folliculin* gene in suspected Birt–Hogg–Dubé syndrome patients

Thomas v. O. Hansen<sup>1</sup>, Anne-Marie Gerdes<sup>2</sup>, Åse Krogh Rasmussen<sup>3</sup>, Lone Sunde<sup>4</sup>, Lennart Friis-Hansen<sup>1</sup>

<sup>1</sup>Dept. of Clinical Biochemistry, Rigshospitalet, University of Copenhagen, Denmark; <sup>2</sup>Dept. of Clinical Genetics, Odense Hospitalet, University of Odense, Denmark; <sup>3</sup>Dept. of Endocrinology, Rigshospitalet, University of Copenhagen, Denmark; <sup>4</sup>Dept. of Clinical Genetics, Århus Hospital, University of Århus, Denmark

**Aim:** To characterize the mutation spectrum in Birt-Hogg-Dubé syndrome patients.

**Method:** Variations were identified by sequencing of the coding region and the intron-exon boundaries of the *Folliculin* (*FCLN*) gene and by analysis of large genomic rearrangements using MLPA.

**Results:** We examined 11 families with clinical symptoms of Birt-Hogg-Dubé syndrome. Mutations were detected in 6 families (54%) and included one splicing mutation (identified in 4 families), one 1-bp deletion in a well-known mutation hot spot (identified in one family) and one missense mutation (identified in one family). Whereas the two former mutations previously have been reported, the missense mutation is novel and the function is unknown. The number of families with the splicing mutation could indicate that it is a Danish founder mutation.

**Conclusion:** We conclude that the mutation spectrum is comparable to that observed in other populations and our study indicates the presence of a Danish *Folliculin* gene founder mutation in Danish Birt-Hogg-Dubé syndrome patients.

### 102

#### Clinical and genetic investigations of Birt–Hogg–Dubé Syndrome at the National Cancer Institute

Jorge R. Toro, Gladys M. Glenn, Ming-Hui Wei, Maria Merino, Peter Choyke, Peter Pinto, Laura S. Schmidt, and W. Marston Linehan

National Cancer Institute, Bethesda, MD, USA

Birt–Hogg–Dubé syndrome (BHDS) (OMIM 135150) is an autosomal dominant predisposition to the development of follicular hamartomas (fibrofolliculomas), lung cysts, spontaneous pneumothorax, and kidney neoplasms. Germline mutations in *FLCN* (also known as BHD) are associated with the susceptibility for BHDS. We previously reported 102 families and 36 unique *FLCN* germline mutations including 21 insertion/deletion, eight putative splice site, six nonsense and one missense mutation types. The mutation detection rate was 84–88%. We reported a 50-fold increased risk for the development of spontaneous pneumothorax in patients with BHDS compared with unaffected family members. Ninety percent of families with members who have BHD germline mutations had lung cysts on screening by chest CT. Kidney tumours were present in 23–34% of individuals with germline *FLCN* mutations. Sixty-five patients with *FCLN* germline mutations and kidney tumours have been reported by the NCI, and to our knowledge only two cases developed metastatic kidney disease. Patients with a germline *FLCN* mutation and family history of kidney cancer had a statistically significant increased probability of developing renal tumors compared to patients without a family history of renal tumors ( $p = 0.0032$ ). Similarly, patients with a *FLCN* germline mutation and family history of spontaneous

pneumothorax had a significantly increased greater probability of having spontaneous pneumothorax than BHDS patients without a family history of spontaneous pneumothorax ( $p = 0.011$ ). Family history of renal cancer and spontaneous pneumothorax are risk factors in BHDS.

## 103

### The folliculin mutation database: an online database of mutations associated with Birt–Hogg–Dubé Syndrome

Matthew Herman, Ming-Hui Wei, Ousmane Toure, Gladys Glenn, Sherri Bale, Jorge R. Toro

National Cancer Institute, Bethesda MD 20852 and GeneDx, Inc, Gaithersburg, MD 20877

Folliculin gene (HGNC approved gene symbol FLCN), also known as BHD is the susceptibility gene for Birt–Hogg–Dubé syndrome (BHDS; OMIM 135150). First described by Birt et al 1977, BHDS is an autosomal dominant predisposition to the development of follicular hamartomas, lung cysts, spontaneous pneumothorax, and kidney neoplasms. FLCN is the only gene known to be associated with BHDS. Sequence analysis detects mutations in the FLCN in 88% of affected individuals and is available on a clinical basis. FLCN encodes a deduced 579-amino acid protein designated folliculin and high sequence conservation was found between human folliculin and homologs in mice, *Drosophila*, and *C. elegans*. The variants included in the database were derived from the published literature and annotated to conform to current mutation nomenclature. The FLCN database applies HGVS nomenclature guidelines, and will assist researchers in applying these guidelines when directly submitting new sequence variants online. Since the first molecular characterization of BHDS, 53 unique pathogenic variant have been reported. In addition, during 2008, my laboratory has identified 10 additional novel variants (manuscript submitted). The majority of FLCN germline mutations are predicted to produce a C-terminally truncated folliculin resulting in loss of function. An online database was constructed detailing all reported FLCN sequence variants. The database strives to systematically unify all current genetic knowledge of FLCN variants. This database will be useful to geneticists, physicians and genetic counselor when counseling patients and their families and provide a rapid and convenient systematic resource to investigators.

## 104

### A case of multiple pulmonary cysts associated with Birt–Hogg–Dubé Gene mutation

Shunsuke Koga, Mitsuko Furuya\*, Yoko Takahashi, Reiko Tanaka, Kazuhiro Yasufuku, Atsushi Yamaguchi, Ichiro Yoshino, Toshio Kumasaka and Yukio Nakatani\*\*

(\*; editorial correspondence, \*\*; corresponding author)

We report a case of multiple pulmonary cysts associated with Birt–Hogg–Dubé (BHD) gene mutation. A 41 year-old Japanese woman with episodes of spontaneous pneumothorax underwent surgical resection of pulmonary cysts in the Department of Thoracic Surgery, Chiba University Hospital. The patient did not have a familial history of pneumothorax. A thorough physical examination excluded the presence of any neoplasms such as a renal tumor and fibrofolliculomas of the skin. Radiological examination showed that multiple cysts were located at atypical sites such as lower lobes and mediastinum vicinities. Pathological analysis of the bullectomy tissues revealed that the luminal surfaces of cysts were lined by pneumocytes. There

was no evidence of endometriosis or lymphangiomyomatosis of the lung. Written informed consent was obtained from the patient and genomic DNA analysis of peripheral blood leukocytes was performed. Direct sequencing of PCR products from all 14 BHD-coding exons predicted a possible gene mutation in exon 12. PCR-SSCP and TA cloning allowed us to identify seven-bases insertion (nt1795 ins CCACCCT) in exon 12, which resulted in frameshift mutation and led to stop codon formation at 16 bases downstream of the mutation site. These results suggested that premature truncation occurred in the C-terminus of BHD gene-encoding protein folliculin, which probably contributed to the development of pulmonary cysts. The significance of BHD gene mutation in pulmonary cysts formation needs further investigation.

Names, e-mail, affiliation, phone and fax numbers of all author

- (1) Shunsuke Koga, E-mail: oshun@lily.ocn.ne.jp
- (2) Mitsuko Furuya\* (editorial correspondence),  
E-mail: mfuruya@yokohama-cu.ac.jp
- (3) Yoko Takahashi, E-mail: ytakahasi@ho.chiba-u.ac.jp
- (4) Reiko Tanaka, E-mail: moru@faculty.chiba-u.jp
- (5) Kazuhiro Yasufuku, E-mail: yasufuku@faculty.chiba-u.jp
- (6) Atsushi Yamaguchi, E-mail: atsuya@restaff.chiba-u.jp
- (7) Ichiro Yoshino, E-mail: yoshino@faculty.chiba-u.jp
- (8) Toshio Kumasaka, E-mail: kumasaka@med.juntendo.ac.jp
- (9) Prof. Yukio Nakatani\*\* (corresponding author)  
E-mail: nakatani@faculty.chiba-u.jp

- (1), (3), (9)\*\* Department of Pathology, Chiba University Graduate School of Medicine, Tel: +81-43-222-7171 ext. 6400, Fax: +81-43-226-2013
- (2)\* Department of Pathology, Yokohama City University Graduate School of Medicine, Tel: +81-45-787-2587, Fax: +81-45-786-0191
- (4) Medical Mycology Research Center, Chiba University, Tel/Fax: 043-226-278
- (5), (7) Department of Thoracic Surgery, Chiba University Graduate School of Medicine, Tel: 043-222-7171 ext. 5464, Fax: 043-226-2172
- (6) Department of Neurobiology, Chiba University Graduate School of Medicine, Tel: 043-226-2024, Fax: 043-226-2025
- (8) Department of Pathology, Juntendo University School of Medicine, Tel: 03-5802-1037, Fax: 03-3812-1056

## 105

### Mutations in BHD in a large series of sporadic chromophobe renal cell carcinomas

Sophie Gad<sup>1</sup>, Sandrine Lefèvre<sup>1</sup>, Sok Kean Khoo<sup>2</sup>, Sophie Giraud<sup>3</sup>, Vincent Molinié<sup>4</sup>, Bin Tean Teh<sup>2</sup> and Stéphane Richard<sup>1,5</sup> on behalf of the French NCI Network “VHL disease and inherited predisposition to kidney cancer”\*

<sup>1</sup>Génétique Oncologique EPHE, CNRS FRE-2939, Institut de Cancérologie Gustave Roussy, 94800 Villejuif, and Faculté de Médecine Paris-Sud, 94270 Le Kremlin-Bicêtre, France; <sup>2</sup>Laboratory of Cancer Genetics, Van Andel Research Institute, Grand Rapids, MI 49503, USA; <sup>3</sup>Laboratoire de Génétique, Hôpital Herriot, 69003 Lyon, France; <sup>4</sup>Laboratoire d'Anatomie Pathologique, Hôpital Saint-Joseph, 75014 Paris, France; <sup>5</sup>Centre Pilote Tumeurs Rares INCa/AP-HP et Consultation d'Oncogénétique Spécialisée, Service d'Urologie, AP-HP, Hôpital de Bicêtre, Le Kremlin-Bicêtre and Service de Néphrologie, AP-HP, Hôpital Necker, Paris, France

\*Main participating members: Annick Vieillefond, Viorel Vasiliu, Yves Denoux, Nicolas Thiounn, Yves Chrétien, Arnaud Méjean,

Marc Zerbib, Gérard Benoît, Jean-Marie Hervé, Brigitte Bressac-de Paillerets.

Corresponding author: Dr Sophie Gad, Laboratoire de Génétique Oncologique EPHE, CNRS FRE-2939, Institut Gustave Roussy, Villejuif-France. Phone: 33 1 42 11 40 11; Fax: 33 1 49 59 67 26; Email: gad@igr.fr

Renal cell carcinoma (RCC) is mainly comprised of clear cell, papillary, and chromophobe subtypes. The germline BHD gene mutations were found mutated at the germline level in patients affected with Birt–Hogg–Dubé (BHD) syndrome that predisposes to various renal tumours (mainly chromophobes RCC and hybrid chromophobe-oncocytic tumours). BHD is a tumour-suppressor gene and somatic inactivation has been reported in BHD-related renal tumours. In sporadic RCC, BHD promoter methylation has been reported in some clear cell and chromophobe RCC but somatic mutation is very rare.

We examined BHD status in a series of 46 sporadic chromophobes RCC by comparison with 19 clear cell RCC, 18 oncocytomas, and 9 papillary RCC. We screened these tumours for mutations and evaluated the promoter methylation status.

Eight mutations were identified in 6 samples: 5 of 46 chromophobe RCC (10.9%) and 1 of 18 oncocytomas (5.6%). Two chromophobe RCC exhibited their respective mutations in both tumoral and corresponding matched normal tissue, suggesting potential germline mutation. One tumour showed loss of the wild-type allele (LOH) but retained its mutant strand in tumoral tissue. Two samples showed a double mutation: one with a germline and a somatic mutations, the other with two distinct somatic mutations. We did not detect any mutation at the hotspot within exon 11 as reported in BHD patients and no evidence of promoter methylation was found.

In summary, this is the first report of somatic BHD mutations in sporadic chromophobe RCC and oncocytomas suggesting that inactivation of BHD is a major event in a subset of these tumours.

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### Familial non-VHL clear cell (conventional) renal cell carcinoma: clinical features, segregation analysis and mutation analysis of FLCN

Emma R Woodward<sup>\*1,2</sup>, Christopher Ricketts<sup>1</sup>, Pip Killick<sup>1</sup>, Sophie Gad<sup>3</sup>, Mark Morris<sup>1</sup>, Fred Kavalier<sup>4</sup>, Shirley V Hodgson<sup>5</sup>, Sophie Giraud<sup>6</sup>, Brigitte Bressac<sup>7</sup>, Cyril Chapman<sup>2</sup>, Bernard Escudier<sup>8</sup>, Farida Latif<sup>1</sup>, Stéphane Richard<sup>3</sup>, Eamonn R Maher<sup>1,2</sup>

\*To whom correspondence should be addressed.

Emma R Woodward, <sup>1</sup>CRUK Renal Molecular Oncology Group and Department of Medical and Molecular Genetics, University of Birmingham, B15 2TT, UK; <sup>2</sup>West Midlands Regional Genetics Service, Birmingham Women's Hospital, B15 2TG, UK; Telephone: +44 121 627 2630/+44 121 627 2741; Fax: +44 121 627 2618; E-mail: E.R.Woodward@bham.ac.uk; Christopher Ricketts, <sup>1</sup>CRUK Renal Molecular Oncology Group and Department of Medical and Molecular Genetics, University of Birmingham, B15 2TT, UK; Telephone: +44 121 414 4452/+44 121 414 3838; Fax: +44 121 414 2538; E-mail: C.J.Ricketts@bham.ac.uk; Pip Killick, <sup>1</sup>CRUK Renal Molecular Oncology Group and Department of Medical and Molecular Genetics, University of Birmingham, B15 2TT, UK; Telephone: +44 121 627 2741; Fax: +44 121 627 2618; E-mail: P.H.Killick@bham.ac.uk; Sophie Gad, <sup>3</sup>Génétique Oncologique EPHE, CNRS FRE-2939, Institut de Cancérologie Gustave Roussy, 94800 Villejuif, and Réseau National INCa "Prédispositions héréditaires au cancer rénal", AP-HP, Service d'Urologie, Hôpital du Kremlin-Bicêtre, 94276 Le Kremlin-Bicêtre, France, Telephone: +33

(0)1 42 11 42 11; E-mail: gad@igr.fr; Mark Morris, <sup>1</sup>CRUK Renal Molecular Oncology Group and Department of Medical and Molecular Genetics, University of Birmingham, B15 2TT, UK; Telephone: +44 121 414 4452/+44 121 414 3838; Fax: +44 121 414 2538; E-mail: M.R.Morris@bham.ac.uk; Fred Kavalier, <sup>4</sup>Department of Clinical Genetics, Guy's Hospital, London SE1 9RT, UK, Telephone: +44 20 7188 1364; Fax: +44 20 7188 1369; E-mail: kavalier@btinternet.com; Shirley V Hodgson, <sup>5</sup>Department of Medical Genetics, St George's University of London, Cranmer Terrace, London, SW17 0RE, UK, Telephone: +44 20 8725 0574; Fax: +44 20 8 725 3444; E-mail: shodgson@sghms.ac.uk; Sophie Giraud, <sup>6</sup>Laboratoire de Génétique, Hôpital Edouard Herriot, 69437 Lyon, France, Telephone: +33 4 72 11 73 83; Fax: +33 4 72 11 73 81; E-mail: sophie.giraud@chu-lyon.fr; Brigitte Bressac, <sup>7</sup>Service de Génétique and CNRS FRE-2939, Institut de Cancérologie Gustave Roussy, 94800 Villejuif, France, Telephone: +33 1 42 11 42 11; E-mail: bressac@igr.fr; Cyril Chapman, <sup>2</sup>West Midlands Regional Genetics Service, Birmingham Women's Hospital, B15 2TG, UK; Telephone: +44 121 627 2630/+44 121 627 6952; Fax: +44 121 627 2618; E-mail: Cyril.Chapman@bwhct.nhs.uk; Bernard Escudier, <sup>8</sup>Département de Médecine, Institut de Cancérologie Gustave Roussy, 94800 Villejuif, France Telephone: +33 1 42 11 42 11; E-mail: escudier@igr.fr; Farida Latif, <sup>1</sup>CRUK Renal Molecular Oncology Group and Department of Medical and Molecular Genetics, University of Birmingham, B15 2TT, UK; Telephone: +44 121 414 4452/+44 121 414 3838; Fax: +44 121 414 2538; E-mail: F.Latif@bham.ac.uk; Stéphane Richard, <sup>3</sup>Génétique Oncologique EPHE, CNRS FRE-2939, Institut de Cancérologie Gustave Roussy, 94800 Villejuif, and Réseau National INCa "Prédispositions héréditaires au cancer rénal", AP-HP, Service d'Urologie, Hôpital du Kremlin-Bicêtre, 94276 Le Kremlin-Bicêtre, France, Telephone: +33 1 42 11 42 11; E-mail: stephane.richard@kb.u-psud.fr; Eamonn R Maher, <sup>1</sup>CRUK Renal Molecular Oncology Group and Department of Medical and Molecular Genetics, University of Birmingham, B15 2TT, UK; <sup>2</sup>West Midlands Regional Genetics Service, Birmingham Women's Hospital, B15 2TG, UK; Telephone: +44 121 627 2630/+44 121 627 2741; Fax: +44 121 627 2618; E-mail: E.R.Maher@bham.ac.uk

Familial renal cell carcinoma (RCC) is genetically heterogeneous. The most common histopathological subtype of sporadic and familial RCC is clear cell (cRCC) and von Hippel-Lindau (VHL) disease is the commonest cause of inherited cRCC. Familial cRCC may also be associated with chromosome 3 translocations and has recently been described in patients with Birt–Hogg–Dubé (BHD) syndrome, caused by germline FLCN mutation. Fewer than 20 kindreds with familial cRCC without VHL disease or a constitutional translocation have been described.

The purpose of this investigation was to define the clinical and genetic features of familial non-VHL cRCC (FcRCC) and to evaluate whether unrecognised BHD syndrome might be present in patients with apparent non-syndromic RCC susceptibility.

We analysed the clinical features of, and undertook segregation analysis in, 60 kindreds containing two or more cases of RCC (at least one confirmed case of cRCC) and no evidence of an RCC susceptibility syndrome. We also undertook FLCN analysis to evaluate whether unrecognised BHD syndrome might be present in 69 patients with apparent non-syndromic RCC susceptibility.

FcRCC was characterised by an earlier age at onset than sporadic cases and more frequent occurrence of bilateral or multicentric tumours. Segregation analysis demonstrated autosomal dominant inheritance with sex and age-dependent penetrance. A germline FLCN mutation was detected in 3/69 (4.3%) patients with apparent non-syndromic RCC susceptibility.

We describe the clinical and genetic features of the largest series of FcRCC and recommend these patients be offered FLCN analysis, in addition to constitutional cytogenetic and VHL analysis.

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### Pathological characterization of pulmonary cysts in Birt–Hogg–Dubé syndrome

Toshio Kumasaka, M.D., Ph.D.<sup>1</sup>, Takuo Hayashi, M.D.<sup>1</sup>, Keiko Mitani, M.T.<sup>1</sup>, Makiko Kunogi, M.D.<sup>2</sup>, Taeko Akiyoshi, B.A.<sup>2</sup>, Yoko Gunji, M.D., Ph.D.<sup>2</sup>, Kuniaki Seyama, M.D., Ph.D.<sup>2</sup>  
 e-mail: kumasaka@med.juntendo.ac.jp, Tkhyz@med.juntendo.ac.jp, m-kei@med.juntendo.ac.jp, komanogi@med.juntendo.ac.jp, akiyosee@med.juntendo.ac.jp, gunji@med.juntendo.ac.jp, kseyama@med.juntendo.ac.jp

Depts. of Human Pathology<sup>1</sup> and Respiratory Medicine<sup>2</sup>, Juntendo University, Graduate School of Medicine, 2-1-1 Hongo, Bunkyo-Ku, Tokyo 113-8421 Japan; Department of Human Pathology, Juntendo University, Graduate School of Medicine. Phone: +81-3-5802-1037, Fax: +81-3-3812-1056; Department of Respiratory Medicine, Juntendo University, Graduate School of Medicine. Phone: +81-3-5802-1063; Fax: +81-3-5802-1617

**Introduction:** BHD syndrome is characterized by hamartoma of the hair follicle, renal tumors, and spontaneous recurrent pneumothorax associated with multiple pulmonary cysts. The purpose of the study was to determine the histopathological features of pulmonary cysts associated with BHD syndrome.

**Material and methods:** Seven cases with BHD syndrome were examined. All cases were women and age ranged 26 to 53 (37.1 + 8.2) years old. Lung tissues were obtained with video-assisted thoracic surgery for the treatment of pneumothorax, inflated and fixed with 10%-buffered formalin. The formalin-fixed paraffin-embedded tissues were sectioned and stained with Hematoxylin-eosin and Elastica-Masson trichrome.

**Results:** A total of thirty-two cysts was analyzed and the diameters of cysts ranged from 2 to 10 mm (7.6 + 1.9 mm). The wall of the cysts comprised pleura, interlobular septa, or normal alveolar walls indicating that the cysts were located at the peripheral area of pulmonary lobule. The characteristic features of the cysts were the pulmonary venules protruded from the cyst wall and the presence of intracystic septa including pulmonary venules. There was no inflammation or fibrosis of the cyst wall except those related with pneumothorax.

**Conclusion:** We found that the histopathologic features of pulmonary cysts associated with BHD syndrome included: (1) cysts located at the peripheral area of pulmonary lobule; (2) venule protruded from or left within cysts; and (3) neither inflammation nor fibrosis in the cyst wall. Further investigations are required to elucidate mechanism of the cyst formation.

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### Is there an increased risk of colorectal cancer in BHD?

L Sunde<sup>1</sup>, L Friis-Hansen<sup>2</sup>, T v O Hansen<sup>2</sup>, Aa K Rasmussen<sup>3</sup>, A-M Gerdes<sup>4</sup>

<sup>1</sup>Department of Clinical Genetics, Aarhus University Hospital, Aarhus, Denmark; <sup>2</sup>Department of Clinical biochemistry, and <sup>3</sup>Department of Endocrinology, Rigshospitalet, Copenhagen, Denmark; <sup>4</sup>Department of Clinical Genetics, Odense University Hospital, Odense, Denmark

**Introduction:** Most carriers of mutations in FLCN manifest fibro-folliculomas and lung cysts, and some develop kidney cancer. Also colon polyps and colon carcinomas have been reported (1,2). However, Zbar et al. (2002) examined members of 33 families with BHD recruited from dermatological specialists, and concluded that BHD-affected individuals did not carry an increased risk of colonic neoplasm.

The cutaneous and pulmonic phenotypes of BHD do not seem to correlate to the genotype. However the risk of renal cancer seems to be low in carriers of the frequent frame-shift mutation c.1733delC.

**Results:** An inquiry to the Danish departments of clinical genetics disclosed 4 families with BHD, verified by detection of mutation in FLCN. In these families a total of 23 persons were either affected, mutation carriers, or both. 3 (13%) of these had a colorectal cancer at age 51, 58, and 59, respectively. Among the affected/mutation carriers above the age of 50, the frequency of colorectal cancer was 27% (3/11). Two of the patients with colorectal cancer carried the mutation IVS9+2T>G, and one c.1733delC.

**Discussion:** Colorectal cancer was observed more frequently in the Danish cohort, than in the cohort examined by Zbar et al. The difference may be caused by chance, as numbers are small. It is also possible that the mode of ascertainment influence the frequencies.

**Conclusion:** Mutation (or some types of mutation) in FLCN may cause an increased risk of colorectal cancer. This hypothesis should be challenged in larger studies, designed in such a way that ascertainment bias is minimized.

1) Le Guyadec et al. *Ann Dermatol Venerol* 1998;123:717

2) Schmidt et al. *Am J Hum Genet* 2005;76:1023

3) Zbar et al. *Cancer Epidemiol Biomarkers Prev* 2002;11:393

e-mail: lsund@as.aaa.dk

Tel: +45 8949 4364

Fax: +45 8949 4370

## Thanks

The organisers would like to thank the committee for all their hard work.

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## Organisations involved with BHD syndrome

The **Birt–Hogg–Dubé Family Alliance** was formed to support all those affected by the Birt–Hogg–Dubé Syndrome. Our goals include spreading awareness of BHD and providing information for families and physicians to aid in diagnosis and treatment of BHD symptoms. We will be publishing online translations of our material in late 2008

at <http://www.birhoggdube.org>; our updated and expanded online material is at <http://www.bhdsyndrome.org>.

The **Myrovlytis Trust** was founded in late 2007 and is based in London. It aims to promote research into BHD syndrome, and its current activities include: funding basic scientific research; supporting the European BHD Consortium; awarding travel grants; organising a recent, small, closed renal gene therapy meeting. See <http://www.myrovlytistrust.org> for further information and contact details.

The **European BHD Consortium** was formed in early 2008 and consists of researchers and clinicians actively working on Birt–Hogg–Dubé syndrome. It currently has members or affiliate members from six countries. The EBC's aims are driven by its ambition to raise awareness of, improve diagnosis of, and facilitate laboratory research into, BHD syndrome. See <http://www.EuropeanBHDConsortium.eu> for further details.