



**HAL**  
open science

## Investigation of the Birt-Hogg-Dubé tumour suppressor gene (FLCN) in familial and sporadic colorectal cancer

Michael Nahorski, Derek Lim, Lynn Martin, Johan Gille, Kirstin Mckay, Pauline Rehal, H. Martijn Ploeger, Maurice van Steensel, Ian Tomlinson, Farida Latif, et al.

### ► To cite this version:

Michael Nahorski, Derek Lim, Lynn Martin, Johan Gille, Kirstin Mckay, et al.. Investigation of the Birt-Hogg-Dubé tumour suppressor gene (FLCN) in familial and sporadic colorectal cancer. *Journal of Medical Genetics*, 2010, 47 (6), pp.385. 10.1136/jmg.2009.073304 . hal-00557374

**HAL Id: hal-00557374**

**<https://hal.science/hal-00557374>**

Submitted on 19 Jan 2011

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

**Investigation of the Birt-Hogg-Dubé tumour suppressor gene (*FLCN*) in familial and sporadic colorectal cancer**

Michael S Nahorski (1), Derek HK Lim(1,2), Lynn Martin (1), Johan JP Gille (3), Kirstin McKay (2), Pauline K Rehal (2), H. Martijn Ploeger (3), Maurice van Steensel (4), Ian P Tomlinson (5), Farida Latif (1), Fred H Menko (3), Eamonn R Maher (1,2)

1. Department of Medical & Molecular Genetics, School of Clinical and Experimental Medicine, University of Birmingham College of Medical and Dental Sciences, Edgbaston, Birmingham B15 2TT, UK

2. West Midlands Regional Genetics Service, Birmingham Women's Hospital, Edgbaston, Birmingham B15 2TG, UK

3. Department of Clinical Genetics, VU University Medical Centre, Amsterdam, the Netherlands

4. Department of Dermatology, Maastricht University Medical Centre and GROW research school for oncology and developmental biology, University of Maastricht, Maastricht, The Netherlands.

5. The Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford, UK

Correspondence to: Prof. E. R. Maher, Department of Medical & Molecular Genetics, School of Clinical and Experimental Medicine, University of Birmingham College of Medical and Dental Sciences, Edgbaston, Birmingham B15 2TT, UK

Tel: (+44) 121 627 2741

Fax: (+44) 121 627 2618

Email: E.R.Maher@bham.ac.uk

## ABSTRACT

Birt–Hogg–Dube´ (BHD) syndrome is an autosomal dominant multisystem disorder with skin (fibrofolliculomas or trichodiscomas), lung (cysts and pneumothorax) and kidney (renal cell carcinoma) tumours. Although colorectal neoplasia was reported initially to be part of the BHD phenotype, some recent studies have not confirmed this association. We undertook a series of clinical and laboratory studies to investigate possible relationships between colorectal neoplasia and the BHD gene (*FLCN*). Thus we investigated whether individuals with familial colorectal cancer of unknown cause might have unsuspected germline *FLCN* mutations, looked for somatic *FLCN* C<sub>8</sub> tract mutations in microsatellite unstable sporadic colorectal cancers and assessed the risk of colorectal neoplasia and possible genotype-phenotype correlations in BHD patients. Although we found previously that germline *FLCN* mutations can be detected in ~5% of patients with familial renal cell carcinoma, we did not detect germline *FLCN* mutations in 50 patients with familial non-syndromic colorectal cancer. Analysis of genotype-phenotype correlations for two recurrent *FLCN* mutations identified in a subset of 51 families with BHD demonstrated a significantly higher risk of colorectal neoplasia in c.1285dupC mutation (within the exon 11 C<sub>8</sub> mononucleotide tract) carriers than in c.610delGCinsTA mutation carriers ( $\chi^2=5.78$  P=0.016). Somatic frameshift mutations in the *FLCN* exon 11 C<sub>8</sub> mononucleotide tract were detected in 23% of sporadic colorectal cancers with microsatellite instability suggesting that *FLCN* inactivation might contribute to colorectal tumourigenesis. Our findings suggest that the previously reported clinical heterogeneity for colorectal neoplasia may reflect allelic heterogeneity and the risk of colorectal neoplasia in BHD syndrome requires further investigation.

## INTRODUCTION

Colorectal cancer (CRC) is the third most common form of cancer in the USA and Europe. Genetic factors have an important role in the pathogenesis of CRC and may be implicated in about a third of cases [1]. The identification of genes for familial colorectal cancer not only enhances clinical management of at risk families but can also provide important insights into the pathogenesis of familial and sporadic forms of CRC. Thus germline mutations in the *APC* tumour suppressor gene cause familial adenomatous polyposis and the *APC* gene is somatically inactivated in >80% of sporadic CRC [2, 3]. Similarly germline mutations in mismatch repair genes (most commonly *MSH2*, *MLH1* and *MSH6*) are associated with Lynch syndrome (hereditary non-polyposis colon cancer syndrome (HNPCC)) which is characterized by the finding of microsatellite instability in colorectal polyps and tumours [4]. Defects in mismatch repair can contribute to cancer development by predisposing to somatic mutations in colorectal cancer suppressor genes that contain short repeat coding sequences [5].

Monogenic forms of colorectal cancer such as familial adenomatous polyposis and Lynch syndrome account for up to 5% of all cases of CRC [6]. Whilst there has been considerable recent progress in the identification of common low penetrance colorectal cancer susceptibility alleles (see Houlston et al 2008 and references within [7]), many cases of familial non-HNPCC clusters of colorectal cancer are unexplained. The delineation of additional inherited disorders associated with CRC susceptibility could lead to more accurate diagnosis of familial CRC and/or provide insights into molecular mechanisms of tumorigenesis in sporadic CRC.

Birt-Hogg-Dube' (BHD) syndrome is a dominantly inherited familial cancer syndrome characterized by the development of benign skin tumours (fibrofolliculomas or

trichodiscomas) on the face and upper body, pulmonary cysts and pneumothorax and renal cell carcinoma (RCC) (see [8] and references within). BHD syndrome is caused by mutations in the folliculin gene (*FLCN*) at 17p11.2 [9-12]. More than 40% of germline *FLCN* mutations are accounted for by a hypermutable mononucleotide tract (C<sub>8</sub>) in exon 11 [13, 14]. BHD displays variable expression and incomplete/age-dependent penetrance and is underdiagnosed. However, molecular genetic analysis enables a diagnosis of BHD to be made in individuals who do not satisfy clinical diagnostic criteria. Recently we detected previously unsuspected germline *FLCN* mutations in ~5% of patients with features of non-syndromic inherited RCC (familial RCC, multiple tumours or early onset) [15]. BHD was described in 1977 and early reports suggested an association with colorectal neoplasia [16-21]. However, in a large study of 111 BHD patients, Zbar *et al* 2002 found no association between BHD and colonic polyps or CRC [22]. Nevertheless, Khoo *et al* 2002 described a large family with BHD in which 6 of 20 affected individuals had developed colonic polyposis and two family members had died of probable gastrointestinal cancer [12]. These observations suggested that some BHD families are at increased risk of colorectal neoplasia and that interfamilial differences might be related to allelic heterogeneity or modifier effects. Several studies have investigated the role of *FLCN* inactivation in colorectal tumorigenesis and somatic mutations in the exon 11 mononucleotide tract in CRC with microsatellite instability were identified in two studies [23, 24]. In order to further evaluate the potential role of folliculin in the pathogenesis of CRC we investigated (a) whether individuals with familial colorectal cancer of unknown cause might have germline *FLCN* mutations, (b) the genotype-phenotype correlations for CRC in BHD patients and (c) the frequency and clinicopathological associations of *FLCN* exon 11 mononucleotide tract mutations in microsatellite unstable CRC.

## PATIENTS AND METHODS

### *Patients and samples*

Three cohorts of patients were studied: (a) blood DNA samples from 50 unrelated patients with familial colorectal cancer and no evidence of familial adenomatous polyposis or germline mismatch repair gene mutations (ascertained for the CORGI study [25]) were analysed for germline *FLCN* mutations; (b) clinical data for colorectal neoplasia (colorectal cancer and adenomatous polyps) status was collected from 149 affected patients (from 51 families) with BHD (either known to have a germline *FLCN* mutation, or if mutation negative, clinically affected according to European BHD Consortium diagnostic criteria [8]). A subset of patients (15 British and Dutch kindreds with two recurrent germline *FLCN* mutations) were analysed in order to identify genotype-phenotype correlations for colorectal neoplasia in BHD syndrome and (c). tumour DNA (extracted from paraffin embedded pathological samples) from 30 patients with microsatellite unstable colorectal cancer was analysed for somatic mutations in the C<sub>8</sub> mononucleotide tract in exon 11 of the *FLCN* gene, and at mononucleotide tracts in the *TGFBR2*, *IGF2R* and *MSH6* genes. Participants gave informed consent; the study was approved by South Birmingham Local Research Ethics Committee and was performed in accordance with the Declaration of Helsinki.

### *Molecular Genetic Analysis*

DNA was extracted from blood using standard methods and from paraffin embedded CRC samples using standard procedures [26]. *FLCN* mutation analysis was performed for all coding exons and exon-intron boundaries by PCR amplification and direct sequencing of the PCR products. Primer sequences are shown in Table 3. To test for the presence of frameshift mutations in MSI tumour samples small range, specific PCR reactions were designed. Primer sequences are shown in Table 4. PCR was performed in 50ul volumes using 20mM MgCl<sub>2</sub>,

200uM of each dNTP, 20pmol primers and 1 unit of Faststart Taq DNA polymerase (Roche). 10ul of product was cleaned using 5 units of Antarctic Phosphatase and 5 units of Exonuclease 1 (New England Biolabs). The sequencing reaction consisted of 4ul of cleaned PCR product, 1x ABI sequencing buffer, 20pmol primer and 0.75ul Big Dye terminator cycle sequencing mix (ABI Applied Biosystems) made up to 10 ul with clean H<sub>2</sub>O. Products were sequenced using an ABI 3730 automated sequencer (ABI Applied Biosystems).

### *Statistical Analysis*

Comparison of tumour characteristics for *FLCN* mutated and non-mutated sporadic colorectal cancer was undertaken using Fishers exact test. Comparison of age-related colorectal neoplasia risks in BHD patients was performed using Kaplan-Meier analysis and log rank testing. Statistical significance was taken at 5%.

## RESULTS

### *FLCN Mutation Analysis in Non-Syndromic Familial Colorectal Cancer and Colorectal Cancer Tumours*

*FLCN* mutation analysis was undertaken in 50 unrelated affected individuals (mean age 52.2 years, range 30-72 years) with familial colorectal cancer (at least one relative with colorectal cancer) without evidence of familial adenomatous polyposis (16 patients had colorectal adenomas but no more than 5) or Lynch syndrome (microsatellite stable tumours and/or no detectable mutation in *MSH2* or *MLH1*). However no *FLCN* mutations were detected (95% CI for *FLCN* mutations = 0 to 8.4%).

### *Genotype-phenotype correlations for colorectal cancer in BHD patients.*

The age related risk of colorectal cancer and colorectal neoplasia (cancer or polyps) was calculated for 149 BHD patients from 51 kindreds. The risk of colorectal cancer and colorectal neoplasia in the BHD patient cohort is shown in figures 1 and 2 respectively. 5 patients had developed a CRC (mean age 57.4 years ;range 48-64 years) and 5 patients had had symptomatic colorectal polyps(s) (mean age 52.0 years ;range 42-68 years).

Germline *FLCN* mutations had been identified in 32 (containing 104 affected individuals/*FLCN* mutation carriers) of the 51 families. Six mutations occurred in two or more kindreds but only two mutations were present in >5 patients. Thus the frameshift mutation, c.1285dupC (formerly known as c.1733insC and c.1740dupC) was present in 37 individuals from 9 families and c.610delGCinsTA (formerly known as c.1065-6delGCinsTA) was present in 32 individuals from 6 families. None of the c.610delGCinsTA mutation carriers developed a colorectal polyp or cancer but 5 individuals with a c.1285dupC mutation developed a colorectal neoplasm (3 of which were malignant). Comparison of colorectal

neoplasia risks in c.1285dupC and c.610delGCinsTA gene carriers revealed a significantly higher risk of colorectal neoplasia in the c.1285dupC mutation carriers ( $\chi^2=5.78$   $P=0.016$ ) (see Figure 3).

### ***FLCN C<sub>8</sub> Mononucleotide Repeat Mutation Analysis in Sporadic Colorectal Cancer Tumours with Microsatellite Instability***

Seven of 30 (23%) CRC with microsatellite instability demonstrated a frameshift mutation within the *FLCN* exon 11 mononucleotide repeat. In 5 cases there was a deletion (c.1285delC) and an insertion (c.1285dupC) was detected in two cases (see Figure 4). Mean % ( $\pm$ standard deviation) of microsatellite instability (i.e. % of microsatellite markers showing instability divided by number of microsatellite markers tested) was similar in *FLCN* C<sub>8</sub> mutated and non-mutated tumours (83.5 ( $\pm$ 8.38) and 64.2 ( $\pm$ 5.86) respectively ( $t=1.57$   $P=0.128$ ). Similarly the mean % of mononucleotide microsatellite instability (tested using BAT25, BAT26 and/or BAT40 [4]) was similar for *FLCN* C<sub>8</sub> mutated and non-mutated tumours (77.8 ( $\pm$ 16.47) and 69.6 ( $\pm$ 8.76) respectively ( $t=0.434$   $P=0.667$ ). Results of MSH2 and MLH1 protein expression (by routine immunohistochemistry) were available for 26 tumours. The frequency of *FLCN* C<sub>8</sub> mutations was significantly higher in tumours that demonstrated loss of MLH1 or MSH2 protein expression than in those with no loss (43% (6/14) and 0% (0/12) respectively ( $P=0.017$ ) (see Table 1). All tumours tested demonstrated a mutation within the A<sub>10</sub> tract in *TGFBR2* and 2/30 (7%) harboured a mutation within the G<sub>8</sub> tract in *IGF2R*. The two tumours with *IGF2R* mutations demonstrated instability for all microsatellite markers tested (6/6 and 7/7 markers). Immunohistochemistry for MSH2 and MLH1 expression was available for one of the *IGF2R* unstable cancers and loss of MLH1 expression was detected. No tumour demonstrated a mutation in both *FLCN* and *IGF2R*. *MSH6* mononucleotide tract mutations were detected in 7/30 (23%) of the colorectal cancers

tested (28.5% and 8% respectively of those with and without MSH2 or MLH1 protein expression loss; P=0.17) (Table 2).

## DISCUSSION

The cumulative lifetime risk of developing colorectal cancer in the USA is about 6% [27]. Assessing precise tumour risks in rare familial cancer syndromes is difficult because of limited number of patients available and possible ascertainment bias. We undertook a retrospective study and none of the colorectal tumours or polyps that were diagnosed in our series were detected as a result of screening asymptomatic individuals. Although the risks of colorectal cancer were higher than in a UK general population cohort (see Figure 1), much larger numbers of patients would be required to obtain statistically significant results and in order to obtain more definitive data on colorectal neoplasia risks in BHD syndrome, we plan to perform a prospective multinational study. However despite the limitations of the current study, it has provided several noteworthy findings. Firstly our results differ from those of Toro et al [28] who did not detect a colorectal neoplasm in 152 patients with BHD syndrome. In a subsequent study, the same group reported 3 colorectal tumours in 111 patients with BHD syndrome, but concluded that this was not statistically significant and that there was not an increased risk of colorectal neoplasia in BHD [22]. Such findings contrast with those of Khoo et al who described a high risk of colorectal neoplasia in a large French family with BHD syndrome [12]. Difference in colorectal risk between different studies and families [11, 21, 27] might result from interfamilial differences in exposure to environmental or genetic modifier effects or *FLCN* allelic heterogeneity (i.e. different mutations in *FLCN* might be associated with differing risks of colorectal cancer). We found that BHD patients with an exon 11 mononucleotide tract mutation had a significantly higher risk of colorectal neoplasia than patients with a c.610\_611delGGinsTA frameshift mutation. In addition, we note that the germline *FLCN* mutation in the high risk family described by Khoo et al (2002) also affected the exon 11 mononucleotide tract (c.1285delC (formerly known as c.1733delC). In the absence of nonsense-mediated mRNA decay the c.1285dupC and c.1285delC mutations

would be predicted to result in a protein with p.His429ProfsX27( lacking 126 amino acids) and p.His429ThrfsX39 (lacking 114 amino acids) respectively. It could be hypothesised that, if p.His429ProfsX27 and p.His429ThrfsX39 are produced in colorectal cells, they might have a dominant negative effect on FLCN function that would not be associated with the c.610delGCinsTA mutation (this is predicted to result in a protein (p.Ala204X) lacking 377 amino acids). Alternatively, although both mutations would be predicted to result in proteins lacking the FNIP1 binding region, folliculin is likely to have multiple functions and so these might be differentially affected by the two different mutant proteins. Nevertheless, we note that 19 patients with exon 11 C<sub>8</sub> frameshift mutations described by Toro et al [14] did not develop colorectal neoplasia and further studies are required to confirm our genotype-phenotype findings in a larger dataset.

Somatic inactivation of familial cancer genes can play a major role in the pathogenesis of sporadic tumours as exemplified by the finding of somatic mutations of *APC* and *VHL* in most colorectal and clear cell RCC respectively [29, 30]. In contrast, mutation analysis of *FLCN* has generally revealed a low frequency of mutations in colorectal cancer. Thus in three studies in which the whole of the *FLCN* coding sequence was analysed in primary CRC the frequency of potential mutations (not involving the C<sub>8</sub> tract) was 0/9 CRC [23], 2/29 CRC (germline p. Arg320Gln and somatic p. Arg392Gly missense substitutions) [24] and 2/30 microsatellite stable CRC (p.S79W and p.A445T) [31]. However none of the four missense variants detected have been identified as germline mutations in BHD patients [32], and so the somatic changes may represent “passenger” rather than “driver mutations”. In view of our finding of an association between colorectal neoplasia risk and a germline c.1285dupC mutation, we proceeded to investigate further whether there might be a link between colorectal neoplasia and exon 11 mononucleotide repeat region mutations. Such mononucleotide repeat regions are known to be hypermutable in microsatellite unstable CRC

and although Kahnoski et al 2003 did not detect *FLCN* C<sub>8</sub> mutations in 8 MSI+ CRC[31], Shin et al 2003 detected C<sub>8</sub> frameshift mutations in 16% (5/32) sporadic MSI+ CRC (2 c.1285dupC and 1 c.1285delC and 1 c.1285delCC)[23]. We found *FLCN* C<sub>8</sub> tract mutations in 23% of MSI+ CRC analysed. In each case, in addition to the frameshift mutation, normal wild-type sequence was also detected. Such an appearance might reflect absence of loss of heterozygosity and sequencing of the *FLCN* coding region in four MSI+ tumours with a *FLCN* C<sub>8</sub> tract mutation did not detect a second truncating mutation. (perhaps suggesting a dominant negative effect) or the presence of normal tissue in the tumour sample. Although the frequency of *FLCN* C<sub>8</sub> mutations in MSI+ CRC was less than in *TGFBR2*, *FLCN* was more frequently mutated than *IGF2R* and the frequency was similar to that for *MSH6*. Somatic mutations in *IGF2R* and *MSH6* have been considered to contribute to tumorigenesis in MSI+ CRC [33, 34]; suggesting that the frequency of *FLCN* C<sub>8</sub> mutations in MSI+ CRC could be consistent with *FLCN* mutations undergoing selection during tumorigenesis.

The identification of genotype-phenotype correlations can provide important insights into the relationship between the specific functions of a disease-associated protein and individual components of the disease phenotype. In such cases the effect of specific mutant proteins on gene function can be studied *in vitro*. However, the function of folliculin is as yet, not well characterised. Baba et al 2006 demonstrated that folliculin interacts with FNIP1 (folliculin interacting protein 1), a poorly characterised protein that binds to 5' AMP-activated protein kinase (AMPK)[35]. It was also reported that FLCN phosphorylation was facilitated by FNIP1, and to be dependent on mTOR and AMPK activity, suggesting a functional relationship between FLCN/FNIP1 and mTOR/AMPK signalling and leading to suggestions that FNIP1 and FLCN may be downstream effectors of AMPK and mTOR, [35]. However, the effect of FLCN inactivation on the mTOR pathway has varied between studies. Thus whereas Baba *et al* (2006) found that a FLCN null RCC cell line has evidence of mTOR

activation, Hartman *et al* (2009) reported lower levels of S6 (an indication of mTOR activity) in cysts and tumours from mice with targeted inactivation of the Bhd gene[36]. Dysregulation of the mTOR pathway has been linked to intestinal tumourigenesis as gastrointestinal polyposis occurs in Cowden syndrome [37] and rapamycin (an inhibitor of mTOR complex 1) therapy suppresses polyp formation in a mouse model of model for human familial adenomatous polyposis [38]. Nevertheless folliculin is likely to be implicated in the regulation of multiple signalling pathways and it may be that the risk of CRC in BHD is related to other pathways. Hence, in order to evaluate the possible functional basis of FLCN genotype-phenotype correlations, it will be necessary to first better characterise the function of the *FLCN* gene product.

Previously we identified clinically unsuspected germline *FLCN* mutations in individuals presenting with features of non-syndromic RCC [15]. However we did not identify any germline *FLCN* mutations in patients with features of non-syndromic CRC. This difference between the involvement of BHD in familial non-syndromic RCC and CRC may have several explanations. Firstly, because BHD is a rare disorder and familial CRC is more frequent than familial RCC it might be necessary to study a much larger group of familial CRC patients in order to identify cases with unsuspected BHD mutations. Secondly, whereas BHD is associated with early onset RCC the mean age of colorectal cancer in our BHD patient series was 57.4 years. Many clinical criteria for the diagnosis of familial CRC cancer risk include a bias for earlier onset tumours (e.g. the Amsterdam criteria for the diagnosis of HNPCC), which would seem to make it less likely that BHD patients might present in this group. As older patients with BHD are more likely to have fibrofolliculomas (enabling a clinical diagnosis of BHD), we suggest that in the absence of a previous medical history or family history of BHD-associated clinical features, the frequency of BHD in patients with features of inherited CRC susceptibility is likely to be very low and *FLCN* mutation analysis

is not indicated. The detection of a genotype-phenotype correlation for colorectal neoplasia risk in BHD provides a potential explanation for the reported heterogeneity in colorectal neoplasia risk in BHD. Although further studies are required to confirm and extend the correlation between *FLCN* mutation type and colorectal neoplasia risk, our findings suggest that when colorectal neoplasia does occur in BHD it does not occur at a very early age. If our findings are confirmed then mutation type might be used to determine CRC risk in BHD syndrome and so need for colonoscopy surveillance. In the meantime we suggest that, in BHD families in which there is a history of colorectal neoplasia, colonoscopic screening should be offered to *FLCN* mutation carriers but, in view of the later age at onset of tumours, this could commence at age 45 years.

#### **ACKNOWLEDGEMENTS**

We thank the Myrovlytis Trust for financial support.

The Corresponding Author has the right to grant on behalf of all authors and does grant on behalf of all authors, an exclusive licence (or non exclusive for government employees) on a worldwide basis to the BMJ Publishing Group Ltd to permit this article (if accepted) to be published in Journal of Medical Genetics and any other BMJPGJL products and sublicences such use and exploit all subsidiary rights, as set out in our licence

(<http://group.bmj.com/products/journals/instructions-for-authors/licence-forms>).

## REFERENCES

- [1] Lichtenstein P, Holm NV, Verkasalo PK, Iliadou A, Kaprio J, Koskenvuo M, et al. Environmental and heritable factors in the causation of cancer--analyses of cohorts of twins from Sweden, Denmark, and Finland. *The New England journal of medicine*. 2000 Jul 13;343(2):78-85.
- [2] Kinzler KW, Nilbert MC, Vogelstein B, Bryan TM, Levy DB, Smith KJ, et al. Identification of a gene located at chromosome 5q21 that is mutated in colorectal cancers. *Science (New York, NY)*. 1991 Mar 15;251(4999):1366-70.
- [3] Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer. *Cell*. 1996 Oct 18;87(2):159-70.
- [4] Lynch HT, de la Chapelle A. Hereditary colorectal cancer. *The New England journal of medicine*. 2003 Mar 6;348(10):919-32.
- [5] Markowitz S, Wang J, Myeroff L, Parsons R, Sun L, Lutterbaugh J, et al. Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. *Science (New York, NY)*. 1995 Jun 2;268(5215):1336-8.
- [6] Aaltonen L, Johns L, Jarvinen H, Mecklin JP, Houlston R. Explaining the familial colorectal cancer risk associated with mismatch repair (MMR)-deficient and MMR-stable tumors. *Clin Cancer Res*. 2007 Jan 1;13(1):356-61.
- [7] Houlston RS, Webb E, Broderick P, Pittman AM, Di Bernardo MC, Lubbe S, et al. Meta-analysis of genome-wide association data identifies four new susceptibility loci for colorectal cancer. *Nature genetics*. 2008 Dec;40(12):1426-35.
- [8] Menko FH, van Steensel MAM, Giraud S, Friis-Hansen L, Richard S, Ungari S, Nordenskjöld M, Hansen TVO, Solly J, Maher ER, Birt-Hogg-Dube syndrome: diagnosis and management. *Lancet Oncology* 2009 in press
- [9] Schmidt LS, Warren MB, Nickerson ML, Weirich G, Matrosova V, Toro JR, et al. Birt-Hogg-Dube syndrome, a genodermatosis associated with spontaneous pneumothorax and kidney neoplasia, maps to chromosome 17p11.2. *American journal of human genetics*. 2001 Oct;69(4):876-82.
- [10] Khoo SK, Bradley M, Wong FK, Hedblad MA, Nordenskjöld M, Teh BT. Birt-Hogg-Dube syndrome: mapping of a novel hereditary neoplasia gene to chromosome 17p12-q11.2. *Oncogene*. 2001 Aug 23;20(37):5239-42.
- [11] Nickerson ML, Warren MB, Toro JR, Matrosova V, Glenn G, Turner ML, 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-Dube syndrome. *Cancer Cell*. 2002 Aug;2(2):157-64.
- [12] Khoo SK, Giraud S, Kahnoski K, Chen J, Motorna O, Nickolov R, et al. Clinical and genetic studies of Birt-Hogg-Dube syndrome. *J Med Genet*. 2002 Dec;39(12):906-12.
- [13] Schmidt LS, Nickerson ML, Warren MB, Glenn GM, Toro JR, Merino MJ, et al. Germline BHD-mutation spectrum and phenotype analysis of a large cohort of families with Birt-Hogg-Dube syndrome. *American journal of human genetics*. 2005 Jun;76(6):1023-33.
- [14] Toro JR, Wei MH, Glenn GM, Weinreich M, Toure O, Vocke C, et al. BHD mutations, clinical and molecular genetic investigations of Birt-Hogg-Dube syndrome: a new series of 50 families and a review of published reports. *J Med Genet*. 2008 Jun;45(6):321-31.
- [15] Woodward ER, Ricketts C, Killick P, Gad S, Morris MR, Kavalier F, et al. Familial non-VHL clear cell (conventional) renal cell carcinoma: clinical features, segregation analysis, and mutation analysis of FLCN. *Clin Cancer Res*. 2008 Sep 15;14(18):5925-30.

- [16] Hornstein OP. Generalized dermal perifollicular fibromas with polyps of the colon. *Hum Genet.* 1976 Jul 27;33(2):193-7.
- [17] Birt AR, Hogg GR, Dube WJ. Hereditary multiple fibrofolliculomas with trichodiscomas and acrochordons. *Archives of dermatology.* 1977 Dec;113(12):1674-7.
- [18] Schachtschabel AA, Kuster W, Happle R. [Perifollicular fibroma of the skin and colonic polyps: Hornstein-Knickenberg syndrome]. *Der Hautarzt; Zeitschrift fur Dermatologie, Venerologie, und verwandte Gebiete.* 1996 Apr;47(4):304-6.
- [19] Schulz T, Hartschuh W. Birt-Hogg-Dube syndrome and Hornstein-Knickenberg syndrome are the same. Different sectioning technique as the cause of different histology. *Journal of cutaneous pathology.* 1999 Jan;26(1):55-61.
- [20] Chung JY, Ramos-Caro FA, Beers B, Ford MJ, Flowers F. Multiple lipomas, angioliipomas, and parathyroid adenomas in a patient with Birt-Hogg-Dube syndrome. *International journal of dermatology.* 1996 May;35(5):365-7.
- [21] Rongioletti F, Hazini R, Gianotti G, Rebora A. Fibrofolliculomas, trichodiscomas and acrochordons (Birt-Hogg-Dube) associated with intestinal polyposis. *Clin Exp Dermatol.* 1989 Jan;14(1):72-4.
- [22] Zbar B, Alvord WG, Glenn G, Turner M, Pavlovich CP, Schmidt L, et al. Risk of renal and colonic neoplasms and spontaneous pneumothorax in the Birt-Hogg-Dube syndrome. *Cancer Epidemiol Biomarkers Prev.* 2002 Apr;11(4):393-400.
- [23] Shin JH, Shin YK, Ku JL, Jeong SY, Hong SH, Park SY, et al. Mutations of the Birt-Hogg-Dube (BHD) gene in sporadic colorectal carcinomas and colorectal carcinoma cell lines with microsatellite instability. *J Med Genet.* 2003 May;40(5):364-7.
- [24] da Silva NF, Gentle D, Hesson LB, Morton DG, Latif F, Maher ER. Analysis of the Birt-Hogg-Dube (BHD) tumour suppressor gene in sporadic renal cell carcinoma and colorectal cancer. *J Med Genet.* 2003 Nov;40(11):820-4.
- [25] Kemp ZE, Carvajal-Carmona LG, Barclay E, Gorman M, Martin L, Wood W, et al. Evidence of linkage to chromosome 9q22.33 in colorectal cancer kindreds from the United Kingdom. *Cancer research.* 2006 May 15;66(10):5003-6.
- [26] Verma L, Kane MF, Brassett C, Schmeits J, Evans DG, Kolodner RD, et al. Mononucleotide microsatellite instability and germline MSH6 mutation analysis in early onset colorectal cancer. *J Med Genet.* 1999 Sep;36(9):678-82.
- [27] Bazensky I, Shoobridge-Moran C, Yoder LH. Colorectal cancer: an overview of the epidemiology, risk factors, symptoms, and screening guidelines. *Medsurg Nurs.* 2007 Feb;16(1):46-51; quiz 2.
- [28] Toro JR, Glenn G, Duray P, Darling T, Weirich G, Zbar B, et al. Birt-Hogg-Dube syndrome: a novel marker of kidney neoplasia. *Archives of dermatology.* 1999 Oct;135(10):1195-202.
- [29] Miyoshi Y, Nagase H, Ando H, Horii A, Ichii S, Nakatsuru S, et al. Somatic mutations of the APC gene in colorectal tumors: mutation cluster region in the APC gene. *Human molecular genetics.* 1992 Jul;1(4):229-33.
- [30] Gallou C, Joly D, Mejean A, Staroz F, Martin N, Tarlet G, et al. Mutations of the VHL gene in sporadic renal cell carcinoma: definition of a risk factor for VHL patients to develop an RCC. *Human mutation.* 1999;13(6):464-75.
- [31] Kahnoski K, Khoo SK, Nassif NT, Chen J, Lobo GP, Segelov E, et al. Alterations of the Birt-Hogg-Dube gene (BHD) in sporadic colorectal tumours. *J Med Genet.* 2003 Jul;40(7):511-5.
- [32] Wei MH, Blake PW, Shevchenko J, Toro JR. The folliculin mutation database: An online database of mutations associated with Birt-Hogg-Dube syndrome. *Human mutation.* 2009 Jun 26.

- [33] Souza RF, Appel R, Yin J, Wang S, Smolinski KN, Abraham JM, et al. Microsatellite instability in the insulin-like growth factor II receptor gene in gastrointestinal tumours. *Nature genetics*. 1996 Nov;14(3):255-7.
- [34] Malkhosyan S, Rampino N, Yamamoto H, Perucho M. Frameshift mutator mutations. *Nature*. 1996 Aug 8;382(6591):499-500.
- [35] Baba M, Hong SB, Sharma N, Warren MB, Nickerson ML, Iwamatsu A, et al. Folliculin encoded by the BHD gene interacts with a binding protein, FNIP1, and AMPK, and is involved in AMPK and mTOR signaling. *Proceedings of the National Academy of Sciences of the United States of America*. 2006 Oct 17;103(42):15552-7.
- [36] Hartman TR, Nicolas E, Klein-Szanto A, Al-Saleem T, Cash TP, Simon MC, et al. The role of the Birt-Hogg-Dube protein in mTOR activation and renal tumorigenesis. *Oncogene*. 2009;28:1594-604.
- [37] Umemura K, Takagi S, Ishigaki Y, Iwabuchi M, Kuroki S, Kinouchi Y, et al. Gastrointestinal polyposis with esophageal polyposis is useful for early diagnosis of Cowden's disease. *World J Gastroenterol*. 2008;14:5755-9.
- [38] Fujishita T, Aoki K, Lane HA, Aoki M, Taketo MM. Inhibition of the mTORC1 pathway suppresses intestinal polyp formation and reduces mortality in *ApcDelta716* mice. *Proceedings of the National Academy of Sciences of the United States of America*. 2008;105:13544-9.
- [39] Lubbe SJ, Webb EL, Chandler IP, Houlston RS. Implications of familial colorectal cancer risk profiles and microsatellite instability status. *J Clin Oncol* 2009;27:2238-2244

## TABLES AND FIGURES

<b>Tumour</b>	<b>Folliculin</b>	<b>MSI</b>	<b>Immunohistochemical analysis</b>
<b>1</b>	<b>no mutation</b>	<b>4 of 4 (3/3BATs)</b>	<b>Loss of MLH1</b>
<b>2</b>	<b>no mutation</b>	<b>4 of 4 (3/3BATs)</b>	<b>n/a</b>
<b>3</b>	<b>no mutation</b>	<b>2 of 4 (1/3BATs)</b>	<b>No loss of MLH1/MSH2</b>
<b>4</b>	<b>mutation</b>	<b>7 of 7 (3/3BATs)</b>	<b>Loss of MLH1</b>
<b>5</b>	<b>no mutation</b>	<b>6 of 6 (3/3BATs)</b>	<b>n/a</b>
<b>6</b>	<b>no mutation</b>	<b>2 of 7 (0/3BATs)</b>	<b>No loss of MLH1/MSH2/MSH6/PMS2</b>
<b>7</b>	<b>no mutation</b>	<b>3 of 7 (2/3BATs)</b>	<b>No loss of MLH1/MSH2/MSH6</b>
<b>8</b>	<b>no mutation</b>	<b>2 of 7 (0/3BATs)</b>	<b>No loss of MLH1/MSH2</b>
<b>9</b>	<b>no mutation</b>	<b>4 of 4 (0/0BATs)</b>	<b>Loss of MLH1</b>
<b>10</b>	<b>mutation</b>	<b>4 of 5 (2/2BATs)</b>	<b>Loss of MLH1/PMS2</b>
<b>11</b>	<b>no mutation</b>	<b>3 of 6 (2/2BATs)</b>	<b>No loss of MLH1/MSH2</b>
<b>12</b>	<b>no mutation</b>	<b>2 of 7 (0/3BATs)</b>	<b>No loss of MLH1/MSH2</b>
<b>13</b>	<b>no mutation</b>	<b>3 of 7 (3/3BATs)</b>	<b>No loss of MLH1/MSH2</b>
<b>14</b>	<b>mutation</b>	<b>7 of 7 (3/3BATs)</b>	<b>Loss of MSH2</b>
<b>15</b>	<b>no mutation</b>	<b>7 of 7 (3/3BATs)</b>	<b>Loss of MLH1</b>
<b>16</b>	<b>no mutation</b>	<b>3 of 7 (3/3BATs)</b>	<b>Loss of MLH1</b>
<b>17</b>	<b>no mutation</b>	<b>4 of 6 (3/3BATs)</b>	<b>n/a</b>
<b>18</b>	<b>no mutation</b>	<b>2 of 4 (1/3BATs)</b>	<b>No loss of MLH1/MSH2</b>
<b>19</b>	<b>no mutation</b>	<b>3 of 7 (0/3BATs)</b>	<b>No loss of MLH1/MSH2</b>
<b>20</b>	<b>no mutation</b>	<b>6 of 7 (3/3BATs)</b>	<b>Loss of MLH1</b>
<b>21</b>	<b>no mutation</b>	<b>7 of 7 (3/3BATs)</b>	<b>No loss of MLH/MSH2</b>
<b>22</b>	<b>no mutation</b>	<b>3 of 7 (3/3BATs)</b>	<b>No loss of MLH1/MSH2</b>
<b>23</b>	<b>mutation</b>	<b>3 of 6 (0/3BATs)</b>	<b>n/a</b>
<b>24</b>	<b>no mutation</b>	<b>5 of 7 (1/3BATs)</b>	<b>Loss of MSH2</b>
<b>25</b>	<b>mutation</b>	<b>5 of 7 (2/3BATs)</b>	<b>Loss of MLH1</b>
<b>26</b>	<b>no mutation</b>	<b>2 of 7 (2/3BATs)</b>	<b>No loss of MLH1/MSH2</b>
<b>27</b>	<b>no mutation</b>	<b>7 of 7 (3/3BATs)</b>	<b>Loss of MLH1/PMS2</b>
<b>28</b>	<b>mutation</b>	<b>Unknown</b>	<b>Loss of MLH1/PMS2</b>
<b>29</b>	<b>mutation</b>	<b>7 of 7 (3/3BATs)</b>	<b>Loss of MLH1</b>
<b>30</b>	<b>no mutation</b>	<b>5 of 7 (3/3BATs)</b>	<b>Loss of MSH2</b>

*Table 1: Details of FLCN mutation status and Immunohistochemical status of mismatch repair proteins, in the MSI+ colorectal tumour DNA samples analysed, where information was available.*

<b>Tumour</b>	<b>FLCN</b>	<b>IGF2R</b>	<b>MSH6</b>	<b>TGFBR2</b>
<b>1</b>	-	-	-	+
<b>2</b>	-	-	-	+
<b>3</b>	-	-	-	+
<b>4</b>	+	-	-	+
<b>5</b>	-	+	+	+
<b>6</b>	-	-	-	+
<b>7</b>	-	-	-	+
<b>8</b>	-	-	-	+
<b>9</b>	-	-	+	+
<b>10</b>	+	-	-	+
<b>11</b>	-	-	-	+
<b>12</b>	-	-	-	+
<b>13</b>	-	-	-	+
<b>14</b>	+	-	-	+
<b>15</b>	-	-	-	+
<b>16</b>	-	-	-	+
<b>17</b>	-	-	+	+
<b>18</b>	-	-	-	+
<b>19</b>	-	-	-	+
<b>20</b>	-	-	-	+
<b>21</b>	-	-	+	+
<b>22</b>	-	-	-	+
<b>23</b>	+	-	-	+
<b>24</b>	-	-	-	+
<b>25</b>	+	-	+	+
<b>26</b>	-	-	-	+
<b>27</b>	-	+	-	+
<b>28</b>	+	-	-	+
<b>29</b>	+	-	+	+
<b>30</b>	-	-	+	+

*Table 2: Mutation profile of the mononucleotide repeat in 4 MSI target genes in 30 MSI colorectal tumours. + indicates mutation, - indicates no mutation*

<b>Exon</b>	<b>Forward Primer</b>	<b>Reverse Primer</b>
<b>4</b>	GCAGGAAGTCCATGGCACC	CCTGAGAAGCAGTCTGTGTC
<b>5</b>	GCTTGAGTTTTCCGAGCTCAG	CCTGTGCTGTGCTGATCTGC
<b>6</b>	GCTGATTTGTGCCAGCTGAC	GCAAGCAAACACGGCTAAGG
<b>7</b>	GGACTGATCCTCCAGGAGTC	GCAAGCAAACACGGCTAAGG
<b>8</b>	GCTGGGTGAGCGTCAGGTTTGC	CGTTCTGGGCTGATTCAGAGC
<b>9</b>	CCATGAAGTATCTTGGGCTG	GCTGTCAAGTCACTTCCTGC
<b>10</b>	CGCCTCCCTGAGAAGATAAG	CACAGCGGTTCTGTGCTG
<b>11</b>	ACAAGCTGGTGTGTGACTGG	TCCACAACCCATGACAGAGA
<b>12+13</b>	CACGGTGGGCTAGCGCAG	CAGCTCCAGGTTTTCTCCAGG
<b>14</b>	GGTGTGGATTCCAGCTCTGC	CCTTGCTGGGACACAGCTCC

*Table 3: Details of primers for FLCN mutation analysis*

<b>Gene</b>	<b>F Primer</b>	<b>R Primer</b>
<b>IGF2R</b>	CCCGAACCAAACCTTGTTTA	ATATGATCCCAGCAGCCTGA
<b>TGFBR2</b>	CCTCGCTTCCAATGAATCTC	TGCACTCATCAGAGCTACAGG
<b>MSH6</b>	CTGATAAAACCCCAAACGA	TAGGCTTTGCCATTTTCTG
<b>FLCN</b>	TCCTCCTCAGACCATGCTTC	GGTCCACTTTGGGCCTGA

*Table 4: Details of Primers for analysis of mononucleotide repeat regions in IGF2R, TGFBR2, MSH6 and FLCN*

## FIGURE LEGENDS

Figure 1: Risk of colorectal cancer (with 95% CIs) in a cohort of 149 BHD patients. For comparison, the risk of colorectal cancer in a UK general population cohort was estimated at 0.1% at age 40 years, 0.8% at age 60 years and 4.9% at age 80 years [39].

Figure 2: Risk for colorectal neoplasia (cancers and polyps) (with 95% CIs) in a cohort of 149 BHD patients

Figure 3: Comparison of colorectal neoplasia risks in BHD patients with c.1285dupC (with 95% CI) and c.610\_611delGCinsTA *FLCN* mutations.

Figure 4: Schematic diagram of electropherograms showing the two mutations detected in 30 microsatellite unstable colorectal cancers, affecting the mononucleotide tract in exon 11 of *FLCN*

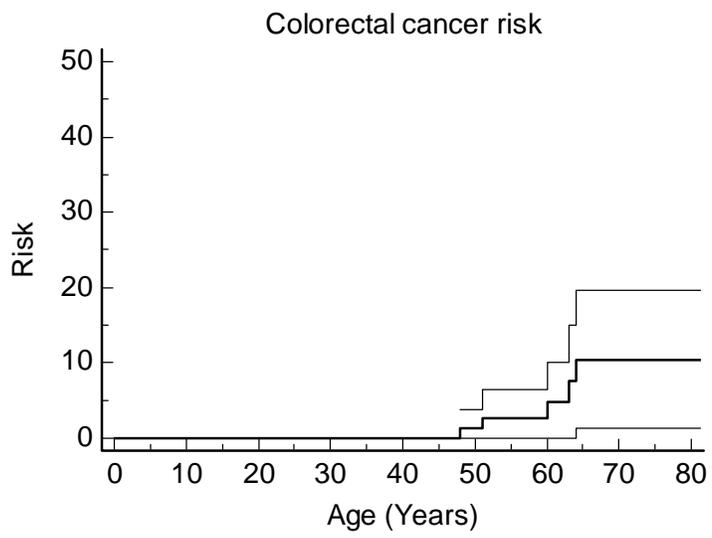


Figure 1

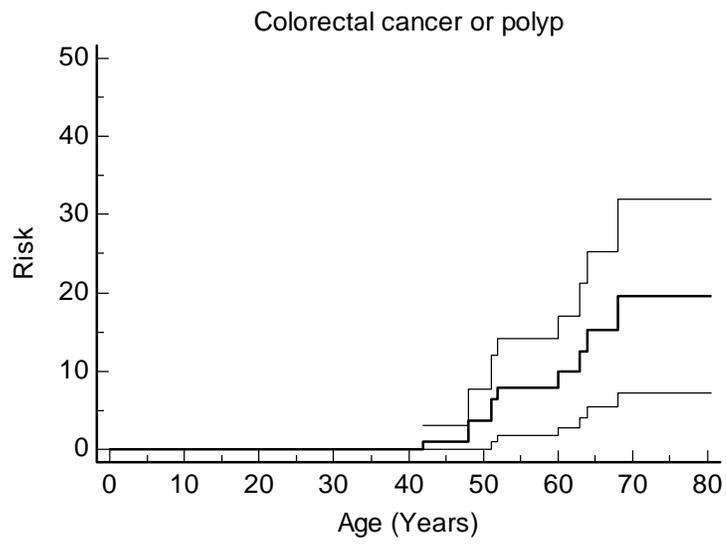


Figure 2

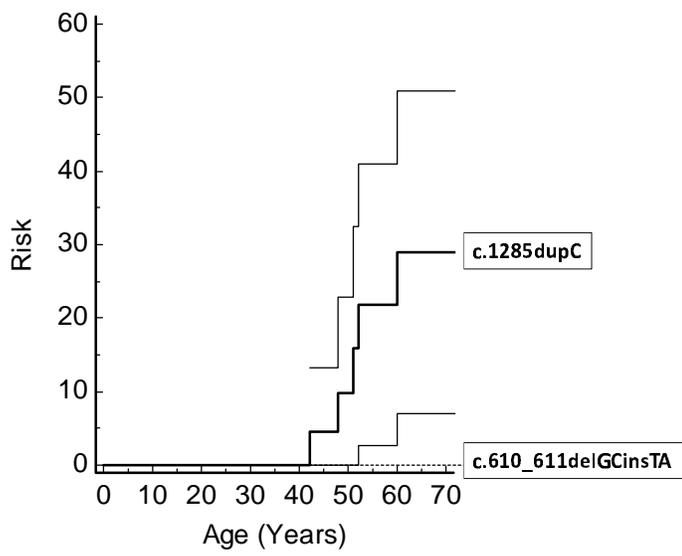
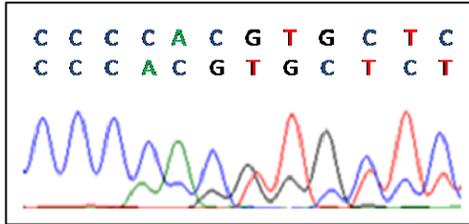


Figure 3

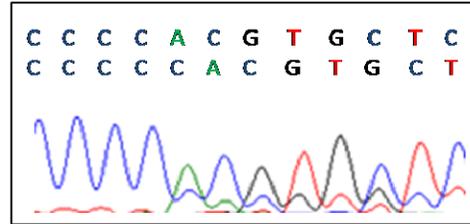
## MSI+ CRC Tumour DNA FLCN EXON 11 (C<sub>8</sub>)

p.Leu429Thrfsx39  
c.1285delC



1bp deletion  
5/30 patients

p.His429Profsx27  
c.1285dupC



1bp duplication  
2/30 patients

Figure 4