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Review

Molecular Pathogenesis of Renal Neoplasms in Patients with Birt–Hogg–Dubé Syndrome

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Abstract: Birt–Hogg–Dubé syndrome (BHDS) is an autosomal dominant disease characterized by skin, lung, and renal manifestations. This syndrome is caused by a germline mutation in the *FLCN* gene, which leads to disruption in multiple downstream pathways. Renal cell carcinomas are one of the serious clinical manifestations of the disease, which usually presents as bilateral and multiple tumors. Morphologically, most of these tumors are classified as hybrid oncocytic tumors. Recent advances in molecular techniques have shed light on the pathogenesis of these renal tumors. In this review, we evaluate and summarize the current knowledge of BHDS, pathologic changes, and its molecular basis with the focus on the renal hybrid oncocytic tumor (HOT), their pathogenesis, and molecular underpinning.

Keywords: Birt-Hogg-Dubé syndrome; RCC; kidney cancer; molecular genetics

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1. Introduction

Renal cell carcinomas (RCCs) accounted for 2% of cancers worldwide in 2020, with an incidence of 431,288 per year [1,2]. While most cases of RCC are sporadic, 4–8% are associated with hereditary syndrome involving causative germline mutation [3–5]. One such hereditary syndrome is Birt–Hogg–Dubé syndrome (BHDS, (OMIM #135150), an autosomal dominant disorder characterized by a constellation of clinical features, including fibrofolliculomas, lung cysts associated, primary spontaneous pneumothorax (PSP), and a markedly increased risk of renal cell carcinoma (RCC). BHDS was first suggested in 1975 by German scientists Hornstein and Knickenberg [6]. However this syndrome is named after three Canadian physicians who studied a family with multiple skin fibrofolliculomas, trichodiscomas, and acrochordons on the head and neck and upper torso in 1977 [7]. Initial publications did not describe extracutaneous manifestations, and the association with renal tumors was not established until 1993 almost twenty years later [8]. In 2001, the genetic basis of BHDS was mapped to chromosome 17p11.2, leading to the identification of mutations in the folliculin (*FLCN*) gene (MIM 607273, formerly called BHD) [9,10]. By 2002, germline mutations in the folliculin gene were identified as the cause of BHDS [11]. These mutations result in a loss of function of the folliculin protein, disrupting multiple cellular metabolic pathways.

BHDS-associated renal cell carcinomas exhibit unusual morphology, and recent research has advanced our understanding of the mechanism underlying BHDS tumorigenesis. This review explores these discoveries, providing a comprehensive overview of BHDS and discusses some unsolved questions as well as our experience. We also examine the clinical implications and potential therapeutic avenues for this group of renal cell carcinomas.

2. BHDS Diagnostic Criteria

The exact prevalence of BHDS remains uncertain, with estimates ranging from 0.5 to 5 per million [12,13]. However, the condition is likely underdiagnosed, and some studies suggest a genetic predisposition as high as 1 in 3000 [14,15].

BHDS exhibits variable expressivity and incomplete penetrance, meaning that clinical presentation of BHDS can differ widely among individuals. Factors influencing expressivity and penetrance are not fully understood but are speculated to include age, race and geographic location, genetic modifiers, environmental factors, and the type of somatic mutations [16,17].

Pulmonary manifestations, such as lung cysts and primary spontaneous pneumothorax due to the rupture of the lung cysts, often precede the development of renal tumors in BHDS patients [18–21]. In some cases, lung cysts and PSP may be the only phenotypic manifestations. Despite this, patients are frequently referred for genetic examination due to the presence of multiple skin tumors.

To address the variability in BHDS manifestation, the European BHD consortium established screening and surveillance guidelines in 2009, incorporating a set of major and minor criteria. The major criterion includes having at least five fibrofolliculomas/trichodiscomas with at least one histologically confirmed and identification of a pathogenic germline variant in the *FLCN* gene. Minor criteria involve the presence of multiple lung cysts, RCC diagnosed before age 50, multiple or bilateral RCC, hybrid RCC, and having a first-degree relative with BHD. Diagnosis requires meeting either one major criterion or two minor criteria [22]. This criteria was later adopted by European Reference Network for patients with a rare genetic tumor risk syndrome (ERN GENTURIS) in 2024, with some modifications to address the increasing use of genetic testing as a first-line diagnostic test [23]. The risk of renal tumors is higher in BHDS patients with a family history of kidney tumors. However, these guidelines have been mostly replaced by molecular genetic testing to detect *FLCN* gene mutations. Genetic testing is the definitive diagnostic tool for BHDS. Methods such as single gene polymerase chain reaction (PCR)-based testing, Sanger sequencing, and Multiple Ligation-dependent Probe Amplification as well as advanced sequencing techniques, like targeted sequencing, whole exome sequencing (WES), and whole genome sequencing (WGS), are employed to identify pathogenic or likely pathogenic variants of the *FLCN* gene.

3. *FLCN* Gene and BHDS Pathogenesis

3.1. FLCN Gene Structure and Normal Expression

The *FLCN* gene (OMIM# 607273) is composed of 14 exons, of which 11 are coding regions encoding the 579-amino acid, 64-kDa protein folliculin. *FLCN* mRNA is expressed in various healthy tissues, including the skin and its appendages, the distal nephron of the kidney, lung stromal cells, and type 1 pneumocytes, and secretory tissues, such as the epithelial cells of the breast, acinar cells of the pancreas, and serous glands of the parotid. There is no expression of *FLCN* in the colon mucinous glands or epithelium [24,25].

3.2. FLCN Gene Mutations

FLCN mutations are predominantly truncating and include duplications (46.4%), deletions (29%), substitutions (7.1), insertions (0.7%), insertion/deletion (0.3%), long genomic deletions (4%), and splice site deletions (12.5%) [26,27]. These mutations lead to early decay or loss of function of the folliculin protein [11,17,20]. Recurrent hotspot mutations account for approximately half of the cases in most populations. Certain regions of the gene, such as hypermutable C8 tract of exon 11, are more prone to mutations due to DNA polymerase slippage during replication. Specific genotypes, such as hypermutable C8 tract mutations, are associated with a higher frequency of certain manifestations, like fewer renal tumors [17,20]. Several founder variants have been reported in Danish, Chinese, and Swedish families [15,28,29].

Efforts to catalog all *FLCN* variants in online databases have enhanced diagnostic and research capabilities in understanding BHDS [27]. A recent systematic literature review identified 1059 individuals with pathogenic *FLCN* variants across 575 families [30].

The online repository of *FLCN* genes maintained by "the Human Variome Project" at Leiden University in the Netherlands lists approximately 230 unique pathogenic and likely pathogenic mutations (http://www.lovd.nl/FLCN, online accessed 17 August 2024).

Somatic *FLCN* mutations occur in other tumors and may predispose individuals to cancer, although the frequency is very low. In a study by Gad et al., somatic *FLCN* mutations were found in 2 of 46 chromophobe renal cell carcinoma (chRCC) and 1 of 18 renal oncocytoma (RO) cases. Other studies have shown similar findings, suggesting that *FLCN* is not a major driver in other renal tumors [31].

While specific genotype–phenotype correlations in BHDS have not been firmly established [17], certain mutations in the *FLCN* gene have been proposed to be related to phenotypes. For example, the c.1285dupC variant is associated with a higher risk of developing renal cancer, and mutations in exon 9 are linked to an increased number of lung cysts and a tendency for PSP [32]. Furthermore, some studies have suggested ethnic variations in the clinical presentation of BHDS. For instance, it is suggested that skin abnormalities are more common in European population, while the Asian population manifests cutaneous symptoms less frequently; Chinese BHDS patients have a higher prevalence of large intragenic deletions spanning exons 1–3, seem to have increased risk of PSP [15,28,29,33–36].

3.3. Mechanism of Action of FLCN

Loss of *FLCN* function due to mutations leads to dysregulation of key cellular pathways such as AMP-activated protein kinase (AMPK) and mechanistic target of rapamycin (mTOR) pathways. This dysregulation causes several downstream effects that promote tumorigenesis, including increased cell proliferation, impaired cellular energy sensing, disrupted autophagy, and altered cellular differentiation.

AMP-activated protein kinase is a positive regulator of catabolic metabolism and a negative regulator of anabolic processes under low energy conditions [37]. FLCN interacts with FLCN-interacting proteins FNIP1 and FNIP2 [38–40], which are involved in the regulation of AMPK and AMPK-mediated energy sensing [41], by forming a complex with FNIP1, FNIP2, and FLCN through binding of the FNIPs to the C-terminal region of *FLCN*. Nearly all mutations in BHDS patients produce a C-terminally truncated FLCN unable to bind FNIP1 [38] (Figure 1).

The mTOR pathway, a major regulator of angiogenesis and cell growth, is rarely mutated but is a downstream effector of frequently mutated oncogenic pathways, including PI3K/Akt and Ras/Raf/Mek/Erk pathways. The Rag GTPases interact with mTORC1 and signal amino acid sufficiency by promoting the translocation of mTORC1 to the lysosomal surface, its site of activation. Structural studies have determined the role of FLCN as a GTPase-activating protein (GAP) for small GTPases, such as Rag GTPases. This GAP activity on the Rags is required for the recruitment of mTORC1 [37,42]. Hyperactivation of mTOR signaling is observed in 80% of human cancers [37]. Under normal conditions, FLCN inhibits the mTOR pathway through AMPK to maintain cellular homeostasis [43]. Downregulation of FLCN reduces the phosphorylation of ribosomal protein S6, an indicator of mTORC1 activity, and disruption in the mTOR signaling pathway results in uncontrolled cell proliferation and growth [37]. However, the role of FLCN/FNIP complex as a positive modulator of mTORC1 activity is controversial, and in certain cell lines, depletion of FLCN impairs mTORC1 activation [42,44].

FLCN loss of function also inhibits mTORC1-dependent phosphorylation of *TFE3*/*TFEB*, resulting in nuclear localization and activation of these transcription factors, which may play a role in tumorigenesis [44,45]. Along the same line of evidence, *PRDM10* alteration has been shown to reduce *FLCN* gene expression, driving *TFE3*-induced tumor formation via canonical mTOR pathway activation. Interestingly, *PRDM10* germline mutations cause a predisposition for a novel hereditary disorder in families with similar manifestations to BHDS, including fibrofolliculomas and renal cancers, but with reduced pulmonary involvement [46,47] (Figure 1).

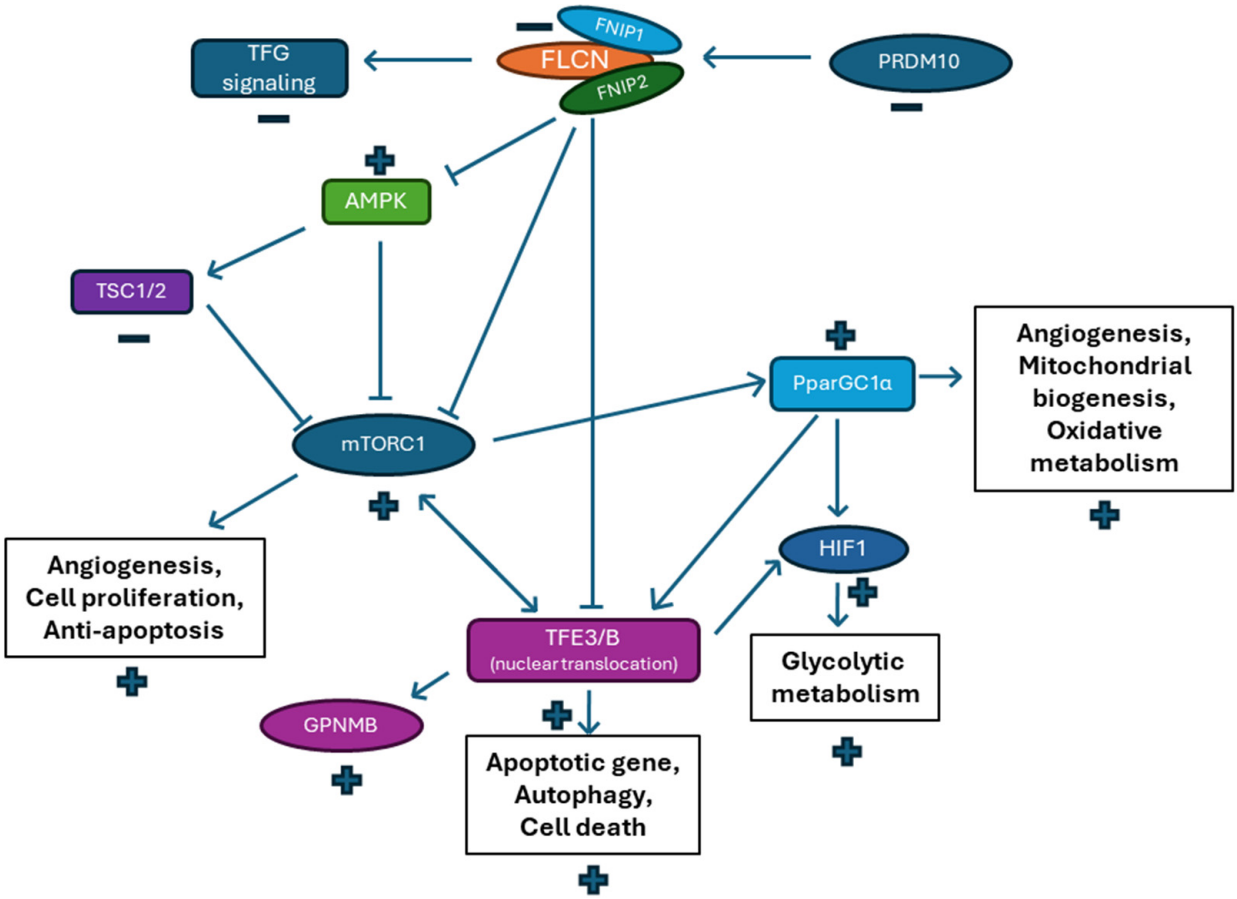

Figure 1. The function of FLCN/FNIP complex mostly occurs through the modulation of mTORC1 and AMPK, two of the key protein kinases. AMPK is an essential player in energetic homeostasis. Loss of FLCN due to *FLCN* gene mutations results in constitutive activation of AMPK. FLCN inhibits the mTOR pathway through AMPK to maintain cellular homeostasis and loss of FLCN leads to mTOR signaling pathway activation and uncontrolled cell proliferation and growth. Subcellular localization of TFE3 and TFEB is controlled by mTORC1. In the absence of FLCN, TFE3 and TFEB translocate to the nucleus and induce the expression of their target genes and induction of HIF pathway. PparGC1α, which is a transcriptional coactivator involved in mitochondrial biogenesis and oxidative metabolism, is also activated by mTORC1.

Loss of *FLCN* induces the upregulation of PPARG coactivator 1 alpha (PPARGC1a) transcription factor, a potent inducer of mitochondrial biogenesis [48]. This leads to deregulation of the PGC-1α-TFAM signaling axis and high expression of mitochondria- and oxidative phosphorylation associated genes [49]. Oxidative phosphorylation provides most of the energy in many somatic cells, whereas malignant transformation generally leads to an increased reliance on glycolysis despite the presence of oxygen (aerobic glycolysis), known as the Warburg effect [50]. FLCN inhibits lactate dehydrogenase A (LDHA) and regulates glycolysis, and pathogenic mutations enable LDHA hyperactivity due to the lack of direct inhibition by FLCN [51]. Loss of FLCN function also leads to AMPK-dependent increases in autophagy, HIF1/2 activity. Interestingly, similar phenomenon is reported in clear cell renal cell carcinoma (ccRCC), where reduced *FLCN* gene expression leads to hyperactive LDHA [51]. Finally, *FLCN* knockdown models in cell lines have been shown to cause impaired autophagy, an evolutionary conserved process of controlled degradation and recycling of damaged organelles and macromolecules [52].

There are rare reports of cases suggesting a combination of BHDS with other syndromes such as Multiple inherited neoplasia alleles syndrome (MINAS) (with multiple rare inherited cancer syndrome genes, including combinations of *FLCN* with *NF1*, *TP53*, *MSH2*,

MLH1, *XPA*, *BRCA2*) or hereditary leiomyomatosis involving BHD [53,54], suggesting a cumulative increase in renal tumor risk. Vocke et al. reported four unrelated adults with Smith–Magenis syndrome (SMS, characterized by a distinctive facial appearance, varying degrees of cognitive impairment and distinct behavioral phenotype) [55] and concomitant features of BHDS [56]. Another case involved a young patient with both BHDS and hereditary paraganglioma-pheochromocytoma syndrome, presenting with metastatic ccRCC and no lung lesions with *FLCN* and *SDHB* germline mutations [57].

3.4. Two-Hit Hypothesis

Individuals with BHDS are born with one variant copy of the *FLCN* gene in each cell. The inactivation of both copies of the *FLCN* gene is a critical step in the development of these tumors. Without somatic second hit mutations, *FLCN* likely exists in a haploinsufficient form, potentially leading to impaired function [58]. Missense germline mutations are rare in BHDS, likely because missense *FLCN* mutations still lead to amino acid substitution that have little or no effect on folliculin function [59]. Heterozygosity for *FLCN* mutations seems sufficient to cause skin and lung lesions, while renal tumors require a second hit in the remaining wild-type allele [60].

Somatic mutations, mostly frameshift mutations or loss of heterozygosity (LOH), inactivate the second copy of the gene [17,61–64]. Second hit *FLCN* alterations may occur in the early third decade of life in BHDS patients [64]. However, some studies report low frequency of LOH in 17p as the second hit [63]. Different second hit mutations have been observed in patients with more than one tumor, supporting the notion that BHDS renal tumors occur independently [61].

3.5. mRNA Expression and Nonsense-Mediated Decay (NMD)

FLCN mRNA expression is hardly detectable in BHD-associated renal tumors [25,65,66], suggesting that degradation by NMD is a surveillance mechanism that eliminates mRNAs with premature termination codons (in all but the last exon of a gene) [67]. However, this view is challenged by some studies that did not find a significant differences in the *FLCN* transcript levels, suggesting incomplete NMD in certain mutations (i.e., c.563delT and c.1489-1490delTG) [13,49,68]. This may be due to a truncating mutation in the last 50 nucleotides of the penultimate exon escaping NMD [69]. Non-truncating mutations in *FLCN* do not disrupt the mRNA splicing pattern, supporting the hypothesis that these mutations impair folliculin function by disrupting the stability of the *FLCN* gene product [20,33].

4. Extrarenal Manifestations of BHDS

4.1. Pulmonary Manifestations of BHDS

Lung cysts and associated PSP are the most prevalent features of BHD. Pulmonary cysts, considered a key risk factor for pneumothorax development, occur in 70–100% of patients, typically forming by the age of 40 [3,17,20,22,70–73]. Most patients (76.9%) present with small pulmonary cysts less than 1 cm in diameter. Histologically these cysts are multiple small intraparenchymal structures rimmed by thin fibrous walls and normal pulmonary parenchyma. The vast majority of cysts are in lower lobes of the lungs [74].

Spontaneous pneumothorax is reported in 32–51% of patients with BHD, representing up to a 50-fold increase compared to general populations [20,30,72,75]. Loss of *FLCN* may lead to the imbalance of cell–cell adhesions and cell polarity, contributing to lung cyst development [76]. It is speculated that up to 5–10% of PSP cases are attributable to underlying BHD [77]. PSP may be caused by mutations in multiple genes including *FBN1*, *COL3A1*, *CBS*, *SERPINA1*, and *TSC1/TSC2* genes [78].

BHDS patients with pneumothorax tend to be older (mean age 42yo) and of normal weight (mean BMI 24.7) [74], which deviates from the usual demographic of PSP patients (younger, tall, and low BMI). Therefore, BHDS should be considered in the differential diagnosis of PSP, especially in the presence of a family history, older age, and normal weight. Recurrent PSP are common and tend to persist throughout life.

4.2. Cutaneous Manifestations of BHSD

Cutaneous lesions are one of the most common phenotypic features of BHDS found in 47–85% of BHDS cases, typically appearing by the third or fourth decade of life. These lesions are characterized by multiple small papules, including fibrofolliculomas, trichodiscomas, and acrochordons [3,17,71,79–83]. Fibrofolliculomas and trichodiscomas are benign tumors of the perifollicular connective tissue and mesodermal portion of the hair disk, occurring as yellowish dome-shaped papules. Fibrofolliculoma and trichodiscoma appear similarly in histology, consisting of hamartomatous hair follicles with cords and thin columns of epithelial components in a fenestrated pattern. It has been suggested that fibrofolliculomas and trichodiscomas represent the same lesion. The main difference between the two is that the former has epithelial cell proliferation emanating from the hair follicle, whereas the latter does not [84]. Acrochordons, also known as skin tags, are benign outgrowths of epidermal and dermal tissue, commonly found on the neck, eyelids, upper chest, and axilla. The treatment of cutaneous manifestations, such as fibrofolliculomas, usually involves ablative laser therapy.

4.3. Other Manifestations

Extensive screening studies of families with BHDS continue to report conflicting results about its associations with different lesions across the body. Conflicting reports about the association of BHDS with colon cancer or colonic polyposis and dysplastic lesions have been reported so far. However, a more recent study on 256 BHDS patients by Sattler et al. showed that 50% of BHDS patients had colorectal polyps, including tubulovillous adenomas with high-grade dysplasia and benign gastric polyps, and a moderately increased rate of colorectal cancer (5.1%) [85]. It has also been suggested that different mutations of the *FLCN* gene might confer varying risks for colorectal polyps and neoplasia in BHDS patients [86].

Other less common manifestations reported in BHDS include oral fibroma, parotid oncocytoma, lipomas, inverted papilloma of the nose, fibrosarcoma of the leg, basal cell carcinoma, squamous cell carcinoma, and lymphoma [83,87–93].

5. Renal Pathology in BHDS

5.1. Prevalence and Behavior

The lifetime risk (20–30%) for development of renal tumors in BHDS patients is much lower than lung involvement or skin involvement (approximately 90%). However, because of the bilateral and multifocal nature, the presence of renal tumors is one of the most serious manifestations in BHD patients. Renal involvement in BHDS manifests as renal cysts and renal tumors. Renal cysts occur in 25–30% of BHDS cases [33,73–75]. the prevalence of renal tumors in BHDS patients ranges from 27–34%, representing a sevenfold increase in risk compared to general population, with the median age of onset being 46–52 years, and no sex predilection [20,72,79,80,94,95]. However, the earliest onset of RCC in a BHDS patient has been reported at age 14 [81]. Renal tumors are often multiple and bilateral, commonly exhibiting a spectrum of histological patterns in the same patient. It was originally reported that the most common forms of renal tumors in BHDS patients are hybrid oncocytic tumors (HOT) (50–67%), chRCC (23–50%), and RO (3%) [79,94–97]. ccRCC and papillary RCC are also reported in BHDS but with much less frequency [24,79,94]. Based on our own evaluation of more than 20 HOTs, they are essentially the same tumor although they may display different histologic patterns, while other tumors such as ccRCC or papillary RCC may be coincidence, rather than related to *FLCN* mutations. More studies are needed to address this question. If second hit *FLCN* mutations are identified in the ccRCC and pRCC, in the absence of the genetic aberrations known to drive these tumors, it would be assumed they are caused by loss of FLCN.

Another interesting kidney-related phenomenon seen in approximately 58% of BHDS patients are oncocytosis, characterized by clusters or cysts of small epithelial oncocytic cells in non-neoplastic tubules, which may contribute to the development of renal tumors [80]. Although oncocytosis is associated with chRCC, most cases are now thought to be related to BHD, driven from principal cells as opposed to intercalated cells in RO and chRCC [98–100]. Interestingly, oncocytosis is present in 50% of HOTs, studies on the cell of origin show that these cells are different from those comprising RO or chRCC, with diffused L1CAM expression and absence of LINC01187 [100].

Metastasis in renal cell carcinoma is rare in BHDS patients, with instances of mortality, but none have been proven to arise from HOT, indicating a more indolent nature [17]. Houweling et al. reported metastasis in 5 out of 14 patients, 3 of which described as renal cell carcinoma with eosinophilic cytoplasm and characteristics of both ccRCC and chRCC [95]. Benusiglio et al. reported metastasis in 4 out of 32 (one of them was hybrid), and Pavlovich et al. reported 2 mortality due to metastasis out of 14 patients with follow up data, 1 histologically designated as ccRCC and 1 as predominantly clear cells with areas of tubular papillary and chromophobe histology [79,95,97].

5.2. HOT Morphology

Hybrid tumors are the most prevalent renal tumors in BHDS patients, notable for their unique morphology and tumorigenesis. The term "hybrid oncocytic tumor (HOT)" has been suggested for hereditary cases seen in Birt–Hogg–Dubé syndrome by the Genitourinary Pathology Society (GUPS) descriptions for renal tumors [101]. These tumors are estimated to have a slow growth rate of 0.1 cm per year [102]. Grossly, HOTs are circumscribed, discrete yellow to tan masses, ranging 0.7 to 5.5 cm, although masses as large as 20 cm have also been reported [81].

Morphologically, the main differential diagnoses include chromophore RCC and RO. HOT often presents with multiple and bilateral lesions, which can be seen in RO but are rare in chRCC. chRCC usually forms nested, alveolar or sheet like patterns and is cytologically distinguished by granular pale cells with prominent cell borders, a finely reticular cytoplasm, perinuclear halos, and wrinkled hyperchromatic nuclei, although it may show deeply eosinophilic features in the oncocytic variant of chRCC [103]. RO, on the other hand, is a benign renal epithelial neoplasm characterized by large round eosinophilic cells with uniformly round hyperchromatic nuclei; smooth nuclear borders very and low nuclear pleomorphism, typically forming small solid nests in a loose connective tissue (edematous-looking) stroma [104,105].

Microscopically, HOT exhibits solid to nested and alveolar/tubular architecture consisting of a checkerboard patterned mixture of RO-like cells with polygonal cells with light eosinophilic cytoplasm and minimal to no koilocytic atypia, with focal edematous stroma, and the second cell population with clear cytoplasm resembling chRCC and round monomorphic nuclei, inconspicuous nucleoli of WHO/International Society of Urological Pathology nucleolar grade 2 and perinuclear halos [64,94,106,107] (Figure 2). The key to correct diagnosis is the two-cell population present in an intermixed pattern, along with multi-locality and bilaterality as well as other syndromic clues. Therefore, a hybrid tumor should not imply that the tumor is a combination of chromophobe cells and renal oncocytoma cells. In our experience with more than 20 cases of HOTs, we have also observed several unique architectural patterns, such as alveolar, solid, slit-like, and microcystic.

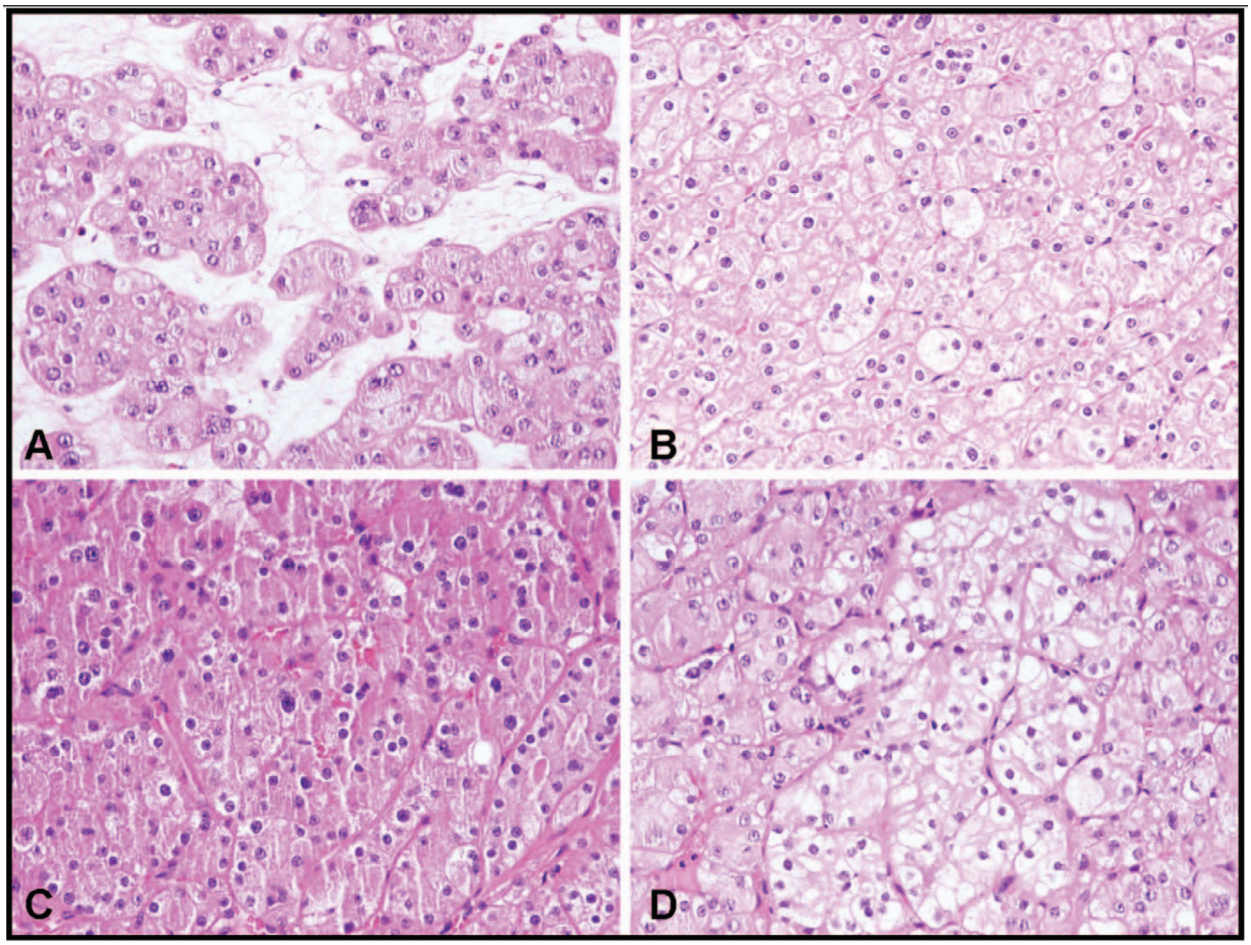

Figure 2. Microscopic features of a hybrid oncocytic tumor (HOT). Most commonly HOTs exhibit a solid nested architecture consisting of a mixture of polygonal cells with light eosinophilic cytoplasm and minimal to no koilocytic atypia resembling renal oncocytoma; and a second cell population with clear cytoplasm resembling chromophobe renal cell carcinoma (chRCC) with perinuclear halos. A predominantly eosinophilic cell nested population may resemble renal oncocytoma (**A**). HOT can resemble chRCC in some areas of the tumor (**B**) and can show a morphology that is not characteristic for renal oncocytoma or chRCC (**C**). An admixture of the two cell populations is characteristic of HOT (**D**). Magnifications 400×. (Reproduced with permission, Adley, B. et al., Arch Pathol Lab Med, 2006) [108].

5.3. Immunohistochemical (IHC) Staining Profile of HOT

The immunohistochemical profile of HOT is ambiguous between RO and chRCC, featuring heterogeneous immunophenotypical cell populations. HOT shows focal positivity for KRT7 and CKIT, and strong PAX8, while being negative for vimentin, and CA9 negative [94,106] (Figure 3). KRT7 is usually diffusely and strongly positive in chRCC, while negative in RO. In HOT, RO-like cells are negative for KRT7 while chRCC-like component is focally positive for KRT7 and diffusely positive for colloidal iron. CKIT is not helpful in distinguishing between these entities. S100A1 has also been reported positive in HOT, which further argues against chRCC. GPNMB (Marker of mTOR pathway activation) IHC stain is strong in the cytoplasm in HOT, but its sensitivity and specificity are not well defined [64,109].

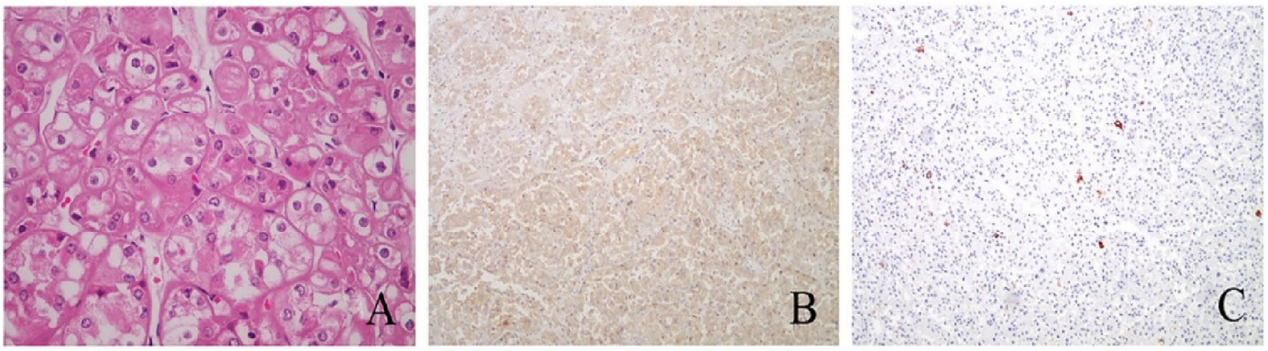

Figure 3. The commonly used immunohistochemical stains for the diagnosis of hybrid oncocytic tumors (**A**), besides positive PAX8 and negative CA9, are weak or negative CD117 (**B**) and patchy positivity for KRT7 (**C**). (Reproduced with permission, Li, J. et al., J Cancer Res Clin Oncol 2022) [110].

5.4. Chromosomal Abnormalities and Mutation Patterns of HOT

HOT exhibits a unique molecular profile distinct from other RCC types. BHD-associated HOTs do not have many recurrent mutations, such as mutations in classic RCC driver genes, such as *VHL*, *BAP1*, *FH*, *MET*, *PTEN*, *TERT*, *TP53*, *ERCC2*, and *SDHA-D* [111,112]. However, they exhibit unique copy number alterations more like RO than chRCC. Comparative gene expression profiling analysis showed that BHDS-related renal tumors have distinct gene expression in BHD-related tumors, with mitochondrial DNA harboring higher copy numbers and fewer variants compared to sporadic chRCC [64]. chRCC is typified by multiple monosomies (1, 2, 6, 7, 10, 13, 17, and 21) and the absence of polysomies [64,112,113]. Sporadic chRCC frequently *TP53* and/or *PTEN* alterations without *FLCN* alteration. RO chromosomal commonly harbors chromosome 1 and Y losses, as well as rearrangement of 11q13, which is the locus of the *CCND1* gene (cyclin D1) [114].

BHD-derived tumors generally exhibit fewer chromosomal abnormalities than sporadic chRCC [49]. Losses are observed in chromosomes 2p, 5p, 8p, 9p, and 19p, which is more similar to RO than chRCC [115]. Single nucleotide polymorphism array studies have shown copious numbers of LOH in BHD-associated renal tumors (classified as chRCC and HOT), mostly due to uniparental disomy, with a similar pattern between two types of tumors [63].

5.5. Two Cell Population in HOT

The origin of the tumor cells in HOT has long intrigued researchers. Recent studies using genomics and single cell sequencing techniques have shown that both chRCC and RO originate from intercalated cells, which have been proven to be different from HOT [64,100,116,117]. RNA transcript data from HOTs suggest an intermediate expression profile between RO and ChRCC [64]. Single cell sequencing data have revealed heterogeneous cell populations with mutually exclusive expression of certain genes [118]. Approximately 50% of the tumor epithelia express FOXI1 and LINC01187, markers of intercalated cells, which are homogeneously expressed in chRCC and RO [64,100,117,119]. The second populations of tumor cells, which are FOXI1-negative, show overexpression of L1CAM and are lineage-specific markers labeling and collecting duct principal cells in the benign kidney. Dual RNA in situ hybridization (RNA-ISH) of LINC1187 and L1CAM demonstrated mutually exclusive expression within HOT epithelia, suggesting that HOT is not a hybrid tumor because of admixture of 'chRCC and RO'-like areas [100]. Instead, HOT exhibits transcriptomic intratumor heterogeneity and displays morphologic and immunophenotypic heterogeneity due to an admixture of neoplastic cells arising from two distinct cells, the IC cells and PC cells [64,117]. This finding suggests that HOT tumors arise from a progenitor cell that is in a state of flux between IC and PC and capable of differentiating into both [100]. During embryologic development, NOTCH signaling regulates the differentiation of the ureteric bud into either L1CAM-positive principal cells or

FOXI1-positive intercalated cells [118]. It is proposed that NOTCH signaling may play a role in the observed heterogeneity of HOT tumors. By validating markers specific for these distinct epithelial populations within the HOT, L1CAM is suggested as the first line diagnostic marker to screen for HOT [100,117,119].

5.6. Clinical Implications and Management

The clinical management of HOTs in BHDS patients requires a tailored approach due to their unique characteristics and the potential for multiple and bilateral tumors and the high chance of recurrence. Given the high risk of renal tumors in BHDS patients, regular surveillance with imaging studies is recommended. Current guidelines suggest initiating surveillance in early adulthood, typically around the age of 20–25 years [22]. Imaging modalities, such as MRI or CT scans, are used to monitor the kidneys for tumor development allowing for timely intervention. Surgical approaches in BHD-associated kidney cancer aim to maximize renal function preservation while preventing metastatic disease. Complex partial nephrectomy is considered the approach of choice for familial kidney cancer syndromes like BHD. The classical protocol includes surveillance of masses of less than 3 cm emphasizing aggressive nephron-sparing techniques for smaller masses while reserving more extensive surgery for larger or more aggressive tumors [102,120,121].

5.7. Therapeutic Approaches

Advances in understanding the molecular pathways involved in BHDS have opened new avenues for targeted therapies. The involvement of the AMPK/mTOR pathway in BHDS pathogenesis suggests that mTOR inhibitors, such as everolimus and sirolimus (routinely used in the treatment of metastatic RCC), may be effective in treating BHD-associated tumors [122,123]. Rapamycin has shown efficacy in halting renal cyst and tumor growth in animal models. Early clinical trials with mTOR inhibitors, such as everolimus, have shown promise, but further research is needed to establish their efficacy and safety in BHDS patients. One study by Nakamura et al. showed that the administration of this drug resulted in longer progression-free survival compared to previously utilized sorafenib and sunitinib [111]. Additionally, understanding the role of autophagy in BHDS tumorigenesis could lead to novel therapeutic strategies targeting this pathway. Recent studies suggest that MET inhibitors, such as cabozantinib and crizotinib, may provide promising therapeutic approaches for BHDS-associated kidney cancer, given the high MET expression in various histological types of BHDS-associated tumors [118].

5.8. Preclinical Models

Developing accurate preclinical models of BHDS is critical for studying the disease and testing potential therapies. Animal models, such as mice with targeted inactivation of the *FLCN* gene, have provided valuable insights into the pathogenesis of BHD-associated tumors. Several *FLCN* knockout mouse models have been created, including skin-specific, lung-specific, muscle-specific, and kidney-specific knockouts. Whole-body *FLCN* knockout and FNIP1/2 double knockout mice are embryonic lethal, indicating a defect in nutrient uptake and transport in the *FLCN*-null embryo [122]. Whole-body *FNIP1* knockout mice, however, show B-cell developmental defects and muscle and cardiac hypertrophy but no kidney phenotype, whereas *FNIP2* knockout mice show no phenotype [124]. Kidney-targeted *FLCN* knockout or *FNIP1*/*FNIP2* double inactivated mice develop enlarged polycystic kidneys and die at 3 weeks of age due to renal failure [41,125,126]. These models have shown that loss of *FLCN* leads to the development of renal cysts and tumors resembling those seen in human BHDS patients [59,127].

Additionally, these models have been instrumental in studying the role of folliculin in various cellular pathways and in identifying potential therapeutic targets. For example, kidney-specific knockout models disrupting the *FLCN* gene in proximal tubules have demonstrated upregulation of mTOR and TGF-β signaling pathways, contributing to renal

tumorigenesis. Treatment with mTOR inhibitors, such as rapamycin or sirolimus, has been shown to suppress tumor growth in these models [128–130].

Other models, such as cell lines derived from BHD-associated tumors, also contribute to understanding the molecular mechanisms underlying BHDS and facilitate the testing of new drugs [131]. RCC cell line models deficient in the *FLCN* protein have shown increased sensitivity to Olaparib treatment, suggesting that *FLCN* deficiency may impair BRCA1-A complex-associated DNA repair ability, thereby making PARP inhibitors potentially more effective in these tumors [132].

6. Discussion

A hybrid oncocytic tumor is a uniquely interesting neoplasm, distinguished by its unique morphology and distinct molecular and tumorigenesis characteristics. In recent years, we have learned that HOT exhibits intrinsic heterogeneity and comprises two distinct cell types. This tumor class is markedly different from sporadic hybrid oncocytic/chromophobe tumors (HOCT), which have been proposed as a heterogeneous group with features intermediate between but distinct from chromophobe RCC and renal oncocytoma [113,133–135]. While sporadic HOCT has been extensively studied, the findings have not been cohesive enough to categorize them as a unified entity. Sporadic HOCTs are characterized by multiple numerical chromosomal aberrations, including both monosomies and polysomies of chromosomes 1, 2, 6, 9, 10, 13, 17, 21, and 22. These tumors lack mutations in key genes such as *VHL*, *KIT*, *PDGFRA*, and *FLCN* [113]. Clinically, these tumors are mostly indolent, the median age of the HOCT patients at the diagnosis is in seventh and eighth decades, are male predominant, and more often present as solitary masses [113,133,136]. These findings put HOCT in a different category compared to BHDS-related HOT. Unlike HOT, the two components of sporadic HOCT are not as intimately intermixed and are typically regarded as separate regions within the tumor, each displaying different morphologies. These findings suggest either two independent pathogenic pathways or an early pathogenic divergence from RO-like and chRCC-like components. Clinically, HOCTs are generally indolent with no evidence of disease recurrence, necrosis, or sarcomatoid change. However, cases of locally advanced disease and metastasis have been reported, indicating potential variability in their clinical behavior.

Transcriptomic analyses of BHD-associated HOT revealed intratumor heterogeneity comprising distinct cell clusters expressing L1CAM and FOXI1, representing two cell populations of intercalated cells (IC) and principal cells (PC). This puts HOT into a very interesting and unique group of tumors, in terms of tumorigenesis. Different models of tumor formation have been proposed. The clonal evolution model suggests that tumors arise from a single cell that acquires a growth advantage. In contrast, the big bang model, proposed in 2015, posits that tumors develop through a single expansion, continuously accumulating mutations not subjected to selective pressure [137]. The neutral evolution model suggests that tumors arise from cells undergoing sequential genomic insults or dedifferentiating into precursor cells with progenitor-like features that can later transform into tumors [138]. Most tumor models assume that mutations accumulate over time, leading to tumor cell dedifferentiation and loss of specialization, but HOT does not seem to perfectly match this model. Recent studies by Wang et al. have demonstrated that HOT originates from progenitor cells capable of differentiating into both IC and PC cells [100], perhaps fitting better in the neutral model and the progenitor-derived hypothesis. This model posits that tumors either lose differentiation into a primitive state or stem/progenitor cells serve as the origin, which contrasts with the course of tumor evolution seen in most other cancers with multiple hit progression. Interestingly, other rare tumors, such as combined hepatocellular-cholangiocarcinoma (cHCC-CCAs), display features of both cholangiocarcinoma and hepatocellular carcinoma in an intermixed pattern. Genomic data suggest a monoclonal origin for both HCC and CCA tumor components in mixed tumors, suggesting a progenitor cell-derived origin. cHCC-CCAs is still a controversial entity, but

its genomic data suggest a monoclonal origin for both HCC and CCA tumor components in mixed tumors [139].

Along the same line of evidence, considering the tumorigenesis and molecular pathways involved in BHDS tumorigenesis, it seems improbable that sporadic chRCC, with its known chromosomal losses, gains, and mutational patterns, would occur alongside HOT in BHDS patients. It is possible that at least some of chRCC cases of BHDS patients in the literature, without confirmation by CGH and molecular confirmatory evidence, may represent a spectrum of HOT, with higher percentage of one cell type as opposed to a balanced distribution. The literature has yet to definitively address this question, although recent RNA-seq data seems to point to this direction, as Jikuya et al. showed that BHD-associated renal tumors display different expression profiles from sporadic chRCC. chRCC does not typically present as multifocal [64]. We recommend that unless chRCC in BHDS is proven to exhibit the classic genetic and unequivocally exhibit classic features of sporadic chRCC without any disputed characteristics, the diagnosis of chRCC should be made with extreme care, as misdiagnosis could lead to unnecessary loss of kidney tissue in BHDS patients, who are prone to developing frequent tumors over time. In a similar fashion, particularly in biopsies, extreme care should be taken not to underdiagnose a potential hybrid tumor as RO. The impact of BHDS extends beyond the affected individuals, with significant implications for genetic counseling and family screening.

Future studies are needed to confirm recent discoveries on cells of origin of HOT and expand the findings presented here. *FLCN* mutations lead to a complex cascade of downstream effects via multiple cellular pathways. The exact mechanisms of these events are not fully understood. Specific mutational signatures (e.g., SBS1, SBS2, and SBS13) differ between chromophobe and BHDS tumors, highlighting the complexity of their genetic landscape [64]. This suggests that epigenetic changes in BHDS may play a role in the heterogeneity of tumors particularly because these tumors do not exhibit extensive mutational and chromosomal changes. By integrating clinical, genetic, and molecular insights, we can improve the diagnosis, management, and treatment of this rare but impactful syndrome.

In conclusion, HOTs in BHDS represent a unique subset of renal tumors with distinct histological, immunohistochemical and molecular features as well as biological behavior. The diagnostic criteria remain to be refined. Accurate diagnosis of these renal tumors can provide the best clinical management strategy and genetic monitoring for the family members who may have BHDS with increased risk of developing renal tumors and other clinical manifestations. Advances in understanding the pathways disrupted by *FLCN* mutations may facilitate the development of targeted therapies, improving outcomes for BHDS patients.

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