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ORIGINAL ARTICLE

Combined germline pathogenic variants in *FLCN* and *TP53* are associated with early onset renal cell carcinoma and brain tumors

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Abstract

Background: We present a family consisting of a father and his two children with an exceptional phenotype of childhood renal cell carcinoma and brain tumors. Extensive genetic testing revealed two inherited tumor predisposition syndromes in all three family members: Birt-Hogg-Dubé syndrome and Li-Fraumeni syndrome. The corresponding genes (*FLCN* and *TP53*) are both located on the short arm of chromosome 17.

Methods: We describe the phenotype and performed single nucleotide polymorphism (SNP)-based loss of heterozygosity (LOH) analysis of the tumors.

Results: All examined tumors showed somatic loss of the wild-type alleles of both *FLCN* and *TP53*.

Conclusions: We hypothesize that a synergistic effect of both mutations caused the unusual phenotype of childhood renal cell carcinoma in this family. This family emphasizes the importance of further genetic testing if a tumor develops at an unexpected young age in an inherited cancer predisposition syndrome.

KEYWORDS

Birt-Hogg-Dubé syndrome, Li-Fraumeni syndrome, loss of heterozygosity, renal cell carcinoma

Maurice A. M. van Steensel, Hendrikus J. Dubbink and Arjan C. Houweling contributed equally to this work.

1 | INTRODUCTION

Birt-Hogg-Dubé syndrome (BHD, MIM #135150) is an autosomal dominant condition caused by pathogenic heterozygous loss of function variants in the tumor suppressor gene *FLCN* (Khoo et al., 2001; Nickerson et al., 2002; Schmidt et al., 2001; Schmidt & Linehan, 2018). Clinical features of BHD are fibrofolliculomas, lung cysts, pneumothorax and renal cell carcinoma (RCC) (Birt et al., 1977; Toro et al., 1999; Zbar et al., 2002). Several subtypes of RCC have been reported in BHD, with the chromophobe carcinoma being the most common (Furuya et al., 2016, 2020; Johannesma et al., 2019; Zbar et al., 2002). A prevalence of renal tumors of 19–34% has been described in several BHD cohorts, but the cumulative risk for RCC until age 70 has been estimated to be 16% (CI 6–26%) (Furuya et al., 2016; Houweling et al., 2011; Schmidt et al., 2005; Toro et al., 2008). The mean age at diagnosis of RCC in BHD is around 50 years (Benusiglio et al., 2014; Furuya et al., 2016; Johannesma et al., 2019; Schmidt et al., 2005). In a Dutch cohort of 199 Dutch BHD patients, 4 out of 23 patients with RCC were diagnosed between the ages of 20 and 30 years (Johannesma et al., 2019). Two of these patients died due to metastatic disease. These findings emphasize the need to start renal surveillance around age 20 as recommended by Menko et al. (2009). The youngest BHD patient with RCC so far was reported in 2018; a 14 year old girl with an unclassifiable RCC (Schneider et al., 2018). Given the early age at diagnosis, extended genetic testing of RCC associated genes and *TP53* was performed but no other possibly pathogenic variants were detected.

Pathogenic germline variants in the tumor suppressor gene *TP53* cause Li-Fraumeni syndrome (LFS, MIM #151623), a hereditary cancer syndrome associated with a high risk for multiple neoplasms at a young age (Li & Fraumeni Jr., 1969; Malkin et al., 1990; Srivastava et al., 1990). A wide variety of malignancies have been reported in LFS. Frequently occurring malignancies are sarcomas, breast cancer, brain tumors, adrenocortical carcinoma and lung cancer (Bougeard et al., 2015). A few patients with LFS and RCC have been reported, of which one occurred in childhood (Curry et al., 2011; Li et al., 1988; Sedlacek et al., 1998). In a large cohort of LFS patients, 4% of the adults and none of the children had a history of renal carcinoma. The mean age at diagnosis of RCC in this LFS cohort was 51 (Bougeard et al., 2015).

Here, we describe a family consisting of a father and his two children with an exceptional phenotype of RCC and brain tumors in childhood. Extensive genetic testing revealed pathogenic germline variants in both *FLCN* and *TP53* in all three patients. These genes both map to the short arm of chromosome 17 and lie around 9.5 Mb apart. Therefore, if no meiotic recombination occurs in between the genes, both variants are inherited together. We show that most tumors lack the whole wild-type 17p arm. We hypothesize that a synergistic effect of both mutations caused the unusual phenotype in this family. These observations might help us to better understand the pathogenesis of RCC in patients with BHD, which is still not completely clarified.

2 | METHODS

2.1 | Patients

Clinical data and histological tissue from a family of three members were collected. Written informed consent for the use of clinical data and histological tissue was obtained. The study was conducted in accordance with the Declaration of Helsinki.

2.2 | Germline genetic testing

All germline genetic testing (Sanger sequencing, MLPA and next generation sequencing [NGS]) and karyotyping was performed in the diagnostic setting in laboratories in the Netherland accredited conform ISO15189.

2.3 | Tumor genetic testing

A single nucleotide polymorphism (SNP)-based loss of heterozygosity (LOH) analysis of the tumors was performed on tumor DNA using targeted, amplicon-based NGS. The exact methods for DNA extraction, NGS, SNP-based LOH testing and data analysis were described previously by Pruis et al. (2020) and Dubbink et al. (2016), respectively. NGS was performed with a pan-cancer diagnostic panel (DiagV4), customized by adding extra amplicons on chromosome 17p (Table S1) and sequence analysis of the coding region of *FLCN* (Table S2).

3 | RESULTS

3.1 | Clinical data

The pedigree is shown in Figure 1. Patient I-1 had undergone treatment for two brain tumors in childhood, which were difficult to classify but most compatible with a pituitary adenoma and an astrocytoma. At age 34 and 35, he was diagnosed with two unclassified

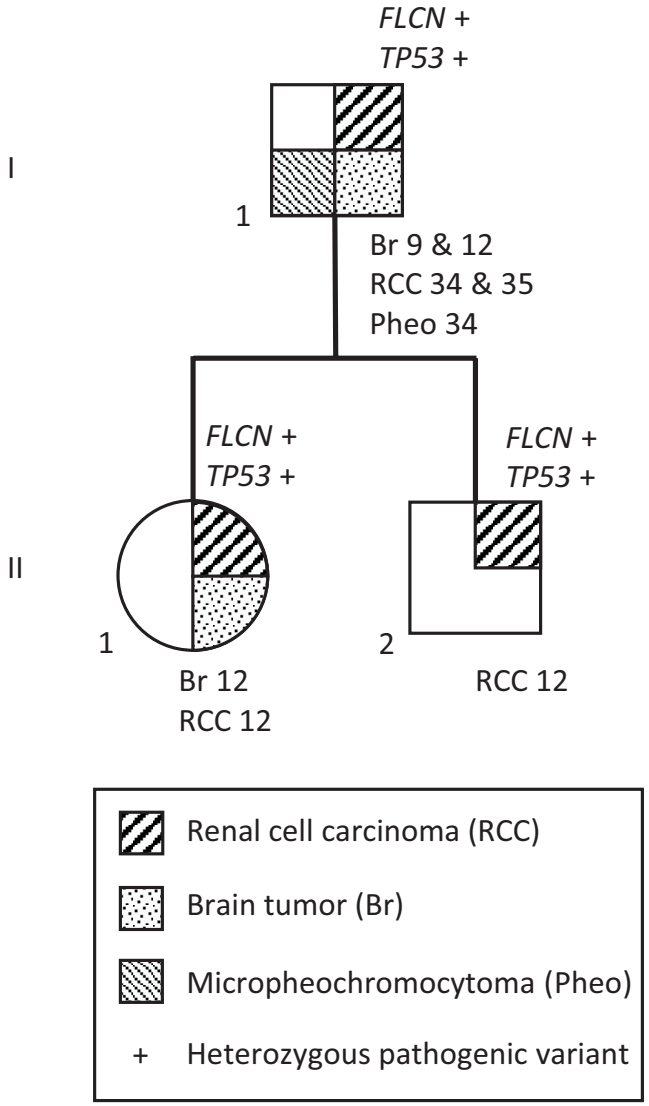

FIGURE 1 Pedigree

RCC in each kidney and a microphaeochromocytoma. In 2005, genetic testing was performed and no (possibly) pathogenic variants were detected in *TSC1, TSC2, RET, VHL* and *MEN1*. Karyotyping showed no chromosomal aberrations. Afterwards, he consulted a dermatologist because of skin lesions. A skin lesion biopsy showed a fibrofolliculoma, which is pathognomonic for BHD. Subsequently, *FLCN* was analyzed and a heterozygous pathogenic variant (c.610_611delGCinsTA, p.[Ala204*]) was detected, which is a relatively common variant among Dutch BHD patients (Houweling et al., 2011). Several years later, his daughter (II-1) was diagnosed with bilateral RCC and a brain tumor at age 12. The RCC's could be classified as a hybrid oncocytic chromophobe tumor and a RCC most compatible with chromophobe carcinoma. Genetic testing had not been performed yet because of her young age and guidelines to start renal surveillance from age 20 in patients with BHD. Her cancer diagnosis prompted genetic testing, which showed that she was a carrier of the familial pathogenic variant in *FLCN*. The young age at diagnosis of her RCC's and her brain tumor was atypical for BHD; therefore, more extensive genetic testing was performed. Since she had a history of a connective tissue nevus removed at age 5, which can be a feature of tuberous sclerosis complex (TSC), genetic testing of *TSC1* and *TSC2* was performed and no pathogenic variants were detected. A clonal relation between the three tumors could not be unambiguously established because in all tumors as well as in normal control tissue from the patient, a pathogenic variant in *TP53* (c.524G > T, p.[Arg175Leu]) was detected. This variant was also detected by subsequent germline testing by Sanger sequencing of the *TP53* gene in DNA extracted from leukocytes. In parallel, germline testing of a hereditary cancer NGS gene panel (114 genes) was performed in her father. This also revealed the pathogenic variant in *TP53*. No other (possibly) pathogenic variants were detected, except for the already known *FLCN* pathogenic variant. Patient II-2 is the younger brother of II-1. Presymptomatic testing showed that he inherited both variants as well. One year afterwards, he was diagnosed with RCC in his left kidney at age 12. An overview of all tumors and their histological classification is shown in Table 1. Representative histological images are shown in Figure 2a,c. No further medical information from other relatives was available. Immunohistochemistry showed aberrant staining of p53 in the different RCC (representative images in Figure 2b,d). The loss of folliculin expression could not be assessed by immunohistochemistry since no specific antibody is available yet.

3.2 | NGS panel analysis

To analyze whether a second hit occurred in *FLCN* and/or *TP53* in the tumors in this family, a SNP-based LOH analysis was performed. No LOH of *FLCN* and/or *TP53* was detected in healthy tissue any of the three patients. Sufficient amounts of DNA were available for somatic analysis of 6 tumors and the connective tissue naevus of II-1 by targeted NGS. The results are shown in Table 1. Five tumors showed somatic loss of the wild-type chromosome 17. One tumor showed allelic imbalance (AI) of the *FLCN* and *TP53* loci but not of the rest of chromosome 17. The connective tissue naevus showed somatic loss of the 17p arm and part of the 17q arm. A visual representation from the LOH analyses in patient II-1 is shown in Figure 3. No other genetic aberrations or mutations were shared by more than 2 tumors (data not shown).

TABLE 1 Tumors and histological data

				NGS panel results					
	Location	**Age at diagnosis**	**Histological description**	***FLCN***	**VAF *FLCN***	***TP53***	**VAF *TP53***	**17p**	**17q**
I-1 (Father)	Brain	9	Difficult classification: most compatible with oncocytic pituitary adenoma	NA	NA	NA	NA	NA	NA
	Brain	12	Difficult classification: most compatible with subependymal giant cell astrocytoma and AV-malformation or angioma	NA	NA	NA	NA	NA	NA
	Left kidney	34	RCC, unclassified, not oncocytic, not chromophobe	NA	NA	NA	NA	NA	NA
	Left kidney	34	RCC, unclassified, not oncocytic, not chromophobe	LOH	81%	LOH	82%	LOH	LOH
	Adrenal gland	34	Micropheochromocytoma	NA	NA	NA	NA	NA	NA
	Right kidney	35	RCC, unclassified, not oncocytic, not chromophobe	NA	NA	NA	NA	NA	NA
	Right kidney	35	RCC, unclassified, not oncocytic, not chromophobe	LOH/AI	59%	LOH/AI	58%	LOH/AI	ROH
	Skin	35	Fibrofolliculoma	NA	NA	NA	NA	NA	NA
II-1 (Daughter)	Skin	5	Connective tissue naevus	LOH	83%	LOH	82%	LOH	LOH partial
	Left kidney	12	RCC, hybrid chromophobe oncocytic	LOH	82%	LOH	80%	LOH	LOH
	Right kidney	12	RCC, most compatible with chromophobe	LOH	70%	LOH	67%	LOH	LOH
	Brain	12	Oncocytic tumor, unclassified	LOH	69%	LOH	67%	LOH	LOH
II-2 (Son)	Left kidney	12	RCC, unclassified, possibly papillary	LOH	73%	LOH	71%	LOH	LOH

Abbreviations: AI, allelic imbalance; LOH, loss of heterozygosity; NA, not assessed; RCC, renal cell carcinoma; VAF, variant allele frequency.

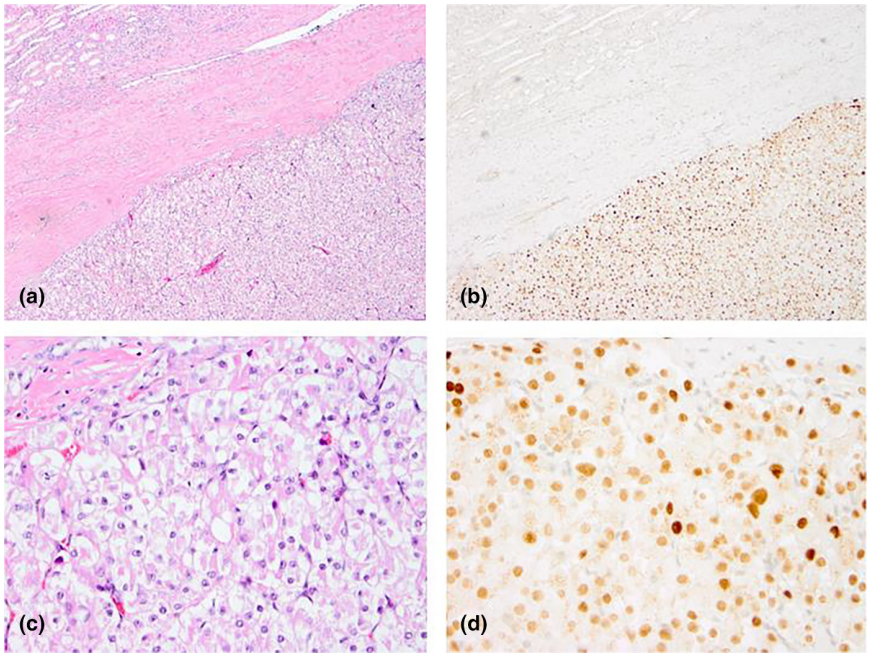

FIGURE 2 Histology and immunohistochemistry of RCC. The renal tumors consisted of compact nests of epithelial cells with granular eosinophilic cytoplasm, large nucleus and conspicuous nucleolus (A, C). Moderate to strong p53 expression (B, D) was observed in all tumor cells, while pre-existent normal renal parenchym (B, top) revealed wild-type expression. Original magnifications A) HE 4x, B) p53 4x, C) HE 20x, D) p53 20x.

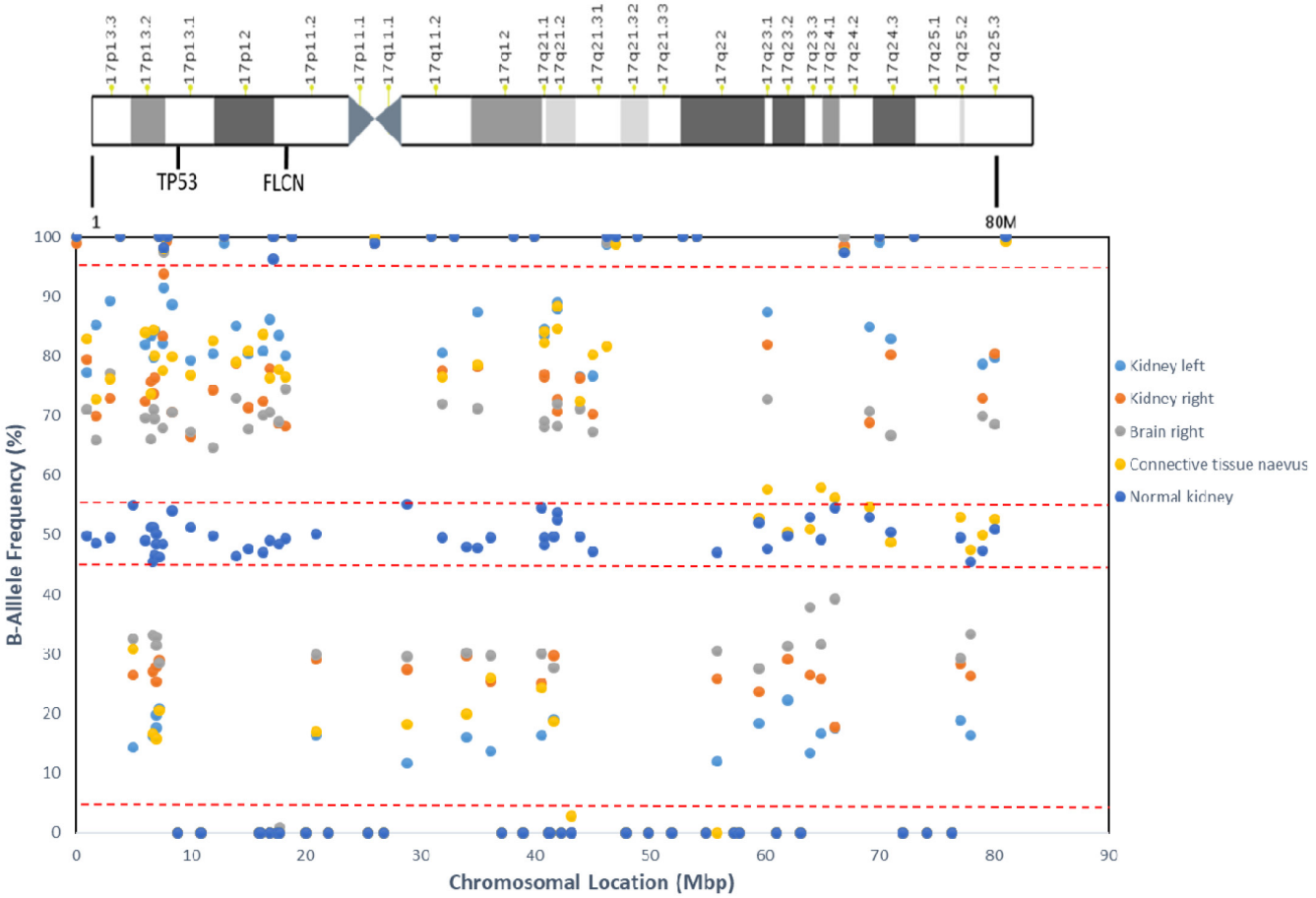

FIGURE 3 SNP-based LOH analysis of chromosome 17 in tumor tissues in patient II-1

4 | CONCLUSIONS

Here, we describe a father and his two children with early onset RCC and brain tumors associated with pathogenic germline variants in both *FLCN* and *TP53*. The co-occurrence of two tumor predisposition syndromes is very rare and the associated tumor risks are often difficult to predict due to the lack of previously

reported cases. Nevertheless, the detection of a second pathogenic variant in a cancer predisposition gene is highly relevant for the affected family, since it allows for genetic testing and tumor surveillance in family members. In addition, establishing a genetic diagnosis can be valuable in making reproductive decisions. Furthermore, clinical and molecular observations in families such as the one reported in the current study may provide further insights into the pathways associated with tumor development and may provide novel targets for therapy. The number of tumors and the age at diagnosis of RCC at age 12 in both children suggests a synergistic effect of both pathogenic variants rather than a result of the two pathogenic variants separately.

The *TP53* pathogenic variant detected here is located in the DNA-binding domain of TP53. As far as we are aware, one case of this variant in the germline was reported in the literature and this was in a child with adrenocortical carcinoma (West et al., 2006). Functional studies show an attenuated tumor suppressor activity of this variant (Dearth et al., 2007; West et al., 2006). The fact that *FLCN* and *TP53* are both located on chromosome 17, could have played a role in the severity of the phenotype in the here described family, since a single event may lead to complete inactivation of both genes. Tumor testing indeed confirmed that all tested tumors harbored LOH of both *FLCN* and *TP53* and most tumors showed unambiguous loss of both wild-type alleles. Even though there are no reports of FLCN and TP53 converging in an overlapping pathway, both genes are tumor suppressor genes and therefore we hypothesized that their complete inactivation underlies the accelerated tumorigenesis observed in this family. Since RCCs are more common in BHD than in LFS, it is most likely that the RCC's in this family are primarily caused by the loss of *FLCN* and that the development is accelerated by the loss of *TP53*. One of the functions of *TP53* is to eliminate cells with cancer-initiating oncogenic lesions (Aubrey et al., 2018). One possible mechanism is that cells with loss of *FLCN* but also loss of *TP53* have a survival benefit compared to cells with loss of *FLCN* only. On the other hand, brain tumors in childhood and young adulthood are common in LFS and not known to be part of the BHD phenotype (Orr et al., 2020). The brain tumors in this family may have been the result of loss of *TP53* on itself or of the combination of both inherited cancer predisposition syndromes. The histological subtypes of the brain tumors in this family are different from the most common subtypes in LFS which are choroid plexus carcinoma, medulloblastoma and astrocytoma (Orr et al., 2020). Interestingly, a connective tissue nevus had been removed in patient II-1 and it also showed somatic loss of both wild-type alleles of *FLCN* and *TP53* (Figure 3). A connective tissue nevus is a hamartoma of the dermis and it is not an established feature of BHD or LFS, but it does occur in TSC (then called shagreen patch). TSC has some phenotypic and molecular overlap with BHD (Arora et al., 2017; Pithadia et al., 2019). In this patient, the connective tissue nevus may also have been the result of the combination of the two inherited tumor syndromes.

So far, one other patient with germline variants in both *FLCN* (c.715C > T, p.[Arg239Cys]) and *TP53* (c.526T > C, p.[Cys176Arg]) has been reported (Whitworth et al., 2016). This male patient was diagnosed with rectal carcinoma at age 27 and gastroesophageal junction adenocarcinoma and chromophobe RCC at age 32. He had skin lesions which were suspect for fibrofolliculomas, but no histological evaluation was reported. Molecular testing of the tumors was not reported. In this case, a synergistic effect of the variants in the two gene is less clear, although the age at diagnosis of RCC in this patient was at a younger age than average in BHD or LFS. Interpretation of the phenotype in this case is impeded by the fact that the c.715C > T variant in *FLCN* variant has now been reported in GnomAD with a high frequency (74 out of 282,778 alleles) (Karczewski et al., 2020). Given the rarity of BHD, it is unlikely that this variant leads to a similar highly penetrant phenotype when compared to truncating pathogenic variants in *FLCN* associated with BHD, even though a phenotype with markedly reduced penetrance cannot be excluded. Therefore, comparing the phenotype of the described patient and our patients with variants in *FLCN* and *TP53* may not be justified and the phenotype in the reported patient could also have been the result of the *TP53* variant alone.

Double heterozygous germline variants of both *TP53* and other tumor predisposition genes have been reported in several patients, for example in combination with variants in *BRCA1*, *BRCA2* and *PTEN* (Bell et al., 2014; Manoukian et al., 2007; Monnerat et al., 2007; Plon et al., 2008). However, some of the reported variants were classified as variants of unknown significance, making it difficult to draw conclusions about the associated phenotypes based on the small number of patients reported. Double heterozygous germline variants of *FLCN* and the tumor predisposition genes *SDHB*, *MSH2* and *NF1* have also been reported (Boland et al., 2020; Whitworth et al., 2016). Some of the phenotypes, such as a tumor not explained by one of the two tumor syndromes separately or a relatively young age at the time of cancer diagnosis, may have been the consequence of the combined variants.

Somatic variants in both *TP53* and *FLCN* are relatively uncommon in sporadic RCC. Pathogenic variants in *TP53* are detected in around 30% of chromophobe RCC and < 10% of clear cell RCC and papillary RCC, while *FLCN* variants only occur in a small percentage of

all RCC subtypes (Cerami et al., 2012; Gao et al., 2013; Liu et al., 2020). Unclassified RCC (uRCC) comprises around 6% of adult RCC and up to 25% of RCC in childhood and young adulthood (Amin et al., 2002; Bruder et al., 2004; Cajaiba et al., 2018; Nguyen et al., 2016; Selle et al., 2006). Not much is known about the molecular characteristics of uRCC and it probably comprises a heterogeneous group of tumors. Molecular analysis of aggressive uRCC has revealed several distinct molecular subsets of uRCC and pathogenic variants in *TP53* were detected in only 5% of uRCC (Chen et al., 2016). RCC in patients with BHD usually has a detectable second hit in *FLCN* and somatic mutations in *TP53* have not been detected in a study of 29 BHD-associated RCC (Hasumi et al., 2018). Taken together, a possible explanation for the high number of uRCC in the family reported here is that their tumors have a different genetic background compared to sporadic RCC, BHD-associated RCC and LFS-associated RCC and therefore distinct histological features.

An animal model could be helpful to test whether the loss of both *FLCN* and *TP53* has a synergistic effect on tumor development. We aimed to study the combined effect of pathogenic variants in *flcn* and *tp53* in a zebrafish model. *Tp53* mutant (M214K) zebrafish develop malignant peripheral nerve sheath tumors (Berghmans et al., 2005). For the purpose of this study, we created *flcn* knockout zebrafish. The homozygous *flcn* knockout zebrafish showed severely impaired growth and a severely shortened life-span of maximum 52 days. The *tp53* status of *flcn* knockout zebrafish did not affect the phenotype. The other way around, the *flcn* mutation status (wild-type or heterozygous) did not affect the *tp53* mutant phenotype (data available upon request). We therefore concluded that the zebrafish might not be the best model to test our hypothesis, mainly because of the severe phenotype of *flcn* knockout and the absence of a phenotype of *flcn* heterozygosity. While a constitutive homozygous deletion of *Flcn* is embryonically lethal in mice, both a heterozygous germline deletion and a kidney-specific *Flcn* knockout results in renal cysts and carcinomas (Chen et al., 2015; Hasumi et al., 2009). Furthermore, mice with heterozygous missense variants in *Trp53* develop a phenotype that is partially similar to LFS in humans and renal carcinoma has occurred in these mice (Lang et al., 2004; Olive et al., 2004). Therefore, the mouse might be a suitable model for future studies.

In conclusion, the family presented here again emphasizes the importance of extensive genetic testing when a genetic diagnosis does not fully explain the observed phenotype. This applies to an unexpected young age of RCC in BHD, but also to other tumor types and/or other inherited cancer syndromes. Furthermore, we conclude that double heterozygous germline pathogenic variants in both *FLCN* and *TP53* may lead to childhood RCC and brain tumors.

AUTHOR CONTRIBUTIONS

Irma van de Beek: designing the study, collection of clinical data, zebrafish experiments, drafting the manuscript. Iris E. Glykofridis: tumor immunohistochemistry, revising the manuscript. Anja Wagner: collection of clinical data, revising the manuscript. Dorine T. den Toom: tumor NGS, revising the manuscript. Ernie M.H.F. Bongers: collection of clinical data, revising the manuscript. Geert J. L. H. van Leenders: collection of clinical data, tumor immunohistochemistry, revising the manuscript. Paul C. Johannesma: collection of clinical data, revising the manuscript. Hanne E.J. Meijers-Heijboer: designing the study, revising the manuscript. Rob M.F. Wolthuis: tumor immunohistochemistry, revising the manuscript. Maurice A.M. van Steensel: designing the study, zebrafish experiments, revising the manuscript. Hendrikus J. Dubbink: designing the study, tumor NGS, tumor immunohistochemistry, revising the manuscript. Arjan C. Houweling: designing the study, collection of clinical data, revising the manuscript.

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CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

This study did not generate/analyze datasets.

ETHICS STATEMENT

Written informed consent for the use of clinical data and histological tissue was obtained. The study was conducted in accordance with the Declaration of Helsinki.

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SUPPORTING INFORMATION

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