Familial multiple discoid fibromas is linked to a locus on chromosome 5 including the FNIP1 gene

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ARTICLE

Familial multiple discoid fibromas is linked to a locus on chromosome 5 including the *FNIP1* gene

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Previously, we reported a series of families presenting with trichodiscomas, inherited in an autosomal dominant pattern. The phenotype was named familial multiple discoid fibromas (FMDF). The genetic cause of FMDF remained unknown so far. Trichodiscomas are skin lesions previously reported to be part of the same spectrum as the fibrofolliculoma observed in Birt-Hogg-Dubé syndrome (BHD), an inherited disease caused by pathogenic variants in the *FLCN* gene. Given the clinical and histological differences with BHD and the exclusion of linkage with the *FLCN* locus, the phenotype was concluded to be distinct from BHD. We performed extensive clinical evaluations and genetic testing in ten families with FMDF. We identified a *FNIP1* frameshift variant in nine families and genealogical studies showed common ancestry for eight families. Using whole exome sequencing, we identified six additional rare variants in the haplotype surrounding *FNIP1*, including a missense variant in the *PDGFRB* gene that was found to be present in all tested patients with FMDF. Genome-wide linkage analysis showed that the locus on chromosome 5 including *FNIP1* was the only region reaching the maximal possible LOD score. We concluded that FMDF is linked to a haplotype on chromosome 5. Additional evaluations in families with FMDF are required to unravel the exact genetic cause underlying the phenotype. When evaluating patients with multiple trichodisomas without a pathogenic variant in the *FLCN* gene, further genetic testing is warranted and can include analysis of the haplotype on chromosome 5.

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INTRODUCTION

Birt-Hogg-Dubé syndrome (BHD) is a multisystem, autosomal dominant disorder characterized by fibrofolliculomas, lung cysts, pneumothorax and an increased risk for renal tumours [1–3]. BHD is caused by pathogenic variants in the *FLCN* gene, encoding folliculin (FLCN) [4]. Fibrofolliculomas are benign skin lesions considered pathognomonic for BHD. Approximately 80–90% of European BHD patients have fibrofolliculomas that are primarily located in the head/neck region and usually develop after the age of 25 [5]. Fibrofolliculomas were initially described as proliferations of specialized connective tissue surrounding an abnormal hair follicle, in which epithelial strands extend out into the connective tissue mantle [2]. In another analysis, fibrofolliculomas were described to consist of branching epithelial strands emerging from the infundibulum of the hair follicle ending in cysts, with prominent association of sebaceous glands [6]. In the past, trichodiscomas have also been considered a characteristic skin lesion in BHD. These are defined as lesions consisting of slightly thickened epithelium overlying a mass of fine fibrillary connective tissue containing prominent blood vessels, with a hair follicle at the edge of the lesion. Later, fibrofolliculomas and trichodiscomas were considered to be different histologic representations of the same lesions observed at different section levels [7–9]. In our opinion, it might be best to consider all hair follicle tumours in BHD as fibrofolliculomas and to consider the trichodiscoma a different lesion not associated with BHD.

In 2012, we reported nine families with trichodiscomas, inheriting in an autosomal dominant pattern [10]. The skin lesions in FMDF were distinguishable from those in BHD by three characteristics. First only trichodiscomas and no fibrofolliculomas were observed in patients with FMDF. Second, the lesions in FMDF were already present in childhood and third, they were primarily localized on the ears. To avoid any confusion with BHD, the disorder was named familial multiple discoid fibromas (FMDF) [10].

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Several other cases of familial trichodiscomas without a pathogenic variant in *FLCN* have been reported since [11–15].

The *FLCN* locus was previously excluded as causal by linkage analysis in the families with FMDF. Given the possible overlap of the skin lesions with those in BHD, we hypothesized that FMDF could be caused by germline mutations in one of the folliculin interacting proteins (FNIP1 and FNIP2) [16–18]. FNIP1 and FNIP2 can bind to the C-terminus of *FLCN* and to *AMP*-activated protein kinase (*AMPK*) [16–19]. *AMPK* plays a role in cellular energy sensing as well as other cellular processes and it is a negative regulator of the mechanistic target of rapamycin (mTOR) [20, 21]. The exact function of FNIP1 in this pathway remains incompletely understood, but most studies have shown an inhibiting effect on AMPK and/or mTOR [22–25]. Furthermore, FLCN is a client protein of the molecular chaperone Hsp90 and FNIP1 acts as a co-chaperone to load FLCN onto Hsp90 [26]. The functions of FLCN and its interaction with FNIP1 and FNIP2 were reviewed recently [27].

Using a candidate gene approach, we identified a variant in *FNIP1* in nine out of ten families with FMDF, while there was no co-segregation with the *FNIP2* locus. We expected the *FNIP1* variant to be causal for FMDF in these families, based on the interaction with *FLCN*, the predicted truncating effect of the variant, and the large number of families in which the variant co-segregated with the FMDF phenotype. Recently however, homozygous and compound heterozygous *FNIP1* truncating variants were reported in children with immunodeficiency, hypertrophic cardiomyopathy and pre-excitation syndrome [28, 29]. No skin lesions were mentioned in these children nor in their heterozygous parents. The parents of one of the reported children were specifically evaluated for a FMDF phenotype, but no skin abnormalities were observed (information kindly provided by dr. Francesco Saettini). We, therefore, decided to perform further comprehensive genetic evaluations in the affected patients. Here, we report the results of our efforts to identify the underlying genetic cause of FMDF.

MATERIALS AND METHODS

Patients

All index patients with FMDF were seen by a clinical geneticist and a dermatologist from the Amsterdam UMC, location VUmc, in the Netherlands and provided informed consent for the study. All but one of the families were previously described in Starink et al. [10]. Family 88 was referred to our outpatient clinic at a later time with similar clinical characteristics as the previously described families. Since many of these families originated from the same village, a common genetic origin was considered. Genomic DNA was isolated from leukocytes from patients and their available unaffected relatives. In 2017, a short questionnaire was sent to all patients asking whether they or their family members developed a pneumothorax or renal cell carcinoma (RCC) since the last clinical evaluation.

Segregation analysis

Segregation analysis for the *FNIP1* and *FNIP2* loci was performed for families 11, 24 and 45, using polymorphic short tandem repeat (STR) markers distal and proximal to both genes. STR markers D4S2394, D4S1644, D4S1625, D4S413, D4S2997, D4S1090, D4S2368, D4S2431, D4S2417, D5S1453, D5S2501, D5S1505, D5S2098, D5S2120, D5S2057, D5S2002, D5S2117, D5S816, D5S1480, D5S820, and D5S1471 were amplified by standard procedures with one of the primers labeled with a fluorescent FAM (carboxyfluorescein) label. Fragment length was analyzed on an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). Haplotype sharing for the other families was evaluated by analyzing all available affected and unaffected family members using markers D5S1505, D5S2098, D5S2120, D5S2057, D5S2002, D5S2117, D5S816, and D5S1480 surrounding the FNIP1 locus.

Sanger sequencing of FNIP1 and PDGFRB

Polymerase chain reaction (PCR) primers were designed to cover all 18 exons and exon/intron boundaries of *FNIP1* (primers available on request). *FNIP1* PCR primers amplifying the fragment harboring NM_133372.3; c.1989del were either Fw 5′ AGATGATTTCTCCTTCTGACTGC 3′ and Rev 5′ ACATTTGTCTACTGGAACAGATCCTG 3′ or Fw 5′ AGCTTACTTAGCTGTGTCAGTAAA 3′ and Rev 5′ CTACTGGAACAGATCCTGTG 3′. *PDGFRB* PCR primers amplifying the fragment harboring NM_002609.3; c.994 C > T were Fw 5′ CCCAGCATCCATATTAGCC 3′ and Rev 5′ GTGGACAGGGCGATTTC 3′. PCR products were sequenced either in-house using the BigDyeTerminator Cycle sequencing kit (Applied Biosystems) and an automated sequencer (ABI 3730 DNA analyzer) or at Eurofins Genomics. Sequencing data was analyzed using Mutation Surveyor software (www.softgenetics.com) and by visual inspection.

Whole exome sequencing

DNA was sheared on a Covaris S2 instrument (Covaris, Woburn, MA). Kapa Biosystems reagents were used to prepare DNA libraries (Kapa Biosystems, Wilmington, MA). Then, 1.0 µg of library was used for enrichment with Roche/NimbleGen KAPA HyperCapture (Roche, Basel, Switzerland) according to the manufacturer's protocol. Whole exome sequencing (WES) was performed with 125 base pair pair-ended reads on an Illumina HiSeq 6000 platform (Illumina, San Diego, CA, USA). Reads were aligned to the human reference genome (GRCh37/Hg19). All shared rare heterozygous variants were analyzed.

Genome-wide linkage analysis

Chromosomal microarray was performed using the Affymetrix CytoScan HD platform (Thermo Fisher Scientific) using standard protocols. We assumed an autosomal dominant mode of inheritance based on the pedigree, the affected status was set according to the data in Fig. 1. The disease allele frequency was set to 0.0001 and penetrance rate was set at either 0.99 or 0.70. Due to the size of the family, approximate multipoint analysis was calculated using the Morgan package [30], run using the Superlink online tool [31].

RESULTS

Clinical evaluation

By genealogical studies, we were able to confirm a common ancestry for eight families with FMDF (Fig. 1). Family 88 was not described before. The family contained multiple family members affected with FDMF. In this family, there was one family member who was said to be affected with FMDF and had a history of renal cancer and colon cancer at age >70 years. The renal cancer concerned a papillary type 2 renal cell carcinoma in an end stage kidney with severe hydronephrosis. It was already reported previously that one individual from family 11 who was said to be affected with FMDF, but not evaluated at the outpatient clinic, had an episode of pneumothorax [10]. Patients VI-8, VII-5, VII-10, VII-11, VIII-9, VIII-10, IX-5 and IX-6 returned the questionnaire and reported that they had no pneumothorax, RCC or other relevant diseases and were not aware of these diagnoses in their relatives.

Detection of the FNIP1 variant

Segregation analysis showed co-segregation of FMDF with the *FNIP1* locus in the three families that were assessed, while there was no co-segregation with the *FNIP2* locus. Further haplotyping showed a shared haplotype at the *FNIP1* locus in nine out of the ten families with FMDF (including the eight families with common ancestry). Sequencing of the *FNIP1* gene revealed a heterozygous germline variant in *FNIP1* (NM_133372.3; c.1989del, p.(Lys664Asnfs*7) in all affected individuals ($n = 24$) and in one unaffected individual. In addition, seven unaffected relatives with an a priori risk of 50% of carrying the *FNIP1* variant were shown not to be carriers of the *FNIP1* variant.

One previously described family with FMDF (family 38) [10] did not have the *FNIP1* variant. Sequencing of the complete *FNIP1* and *FNIP2* genes did not reveal any other possibly pathogenic variants in this family. We observed histological and morphological differences of the trichodiscomas in this family when compared to the other FMDF families. This family may therefore have another inherited cause for their phenotype. No consent for further genetic testing was available.

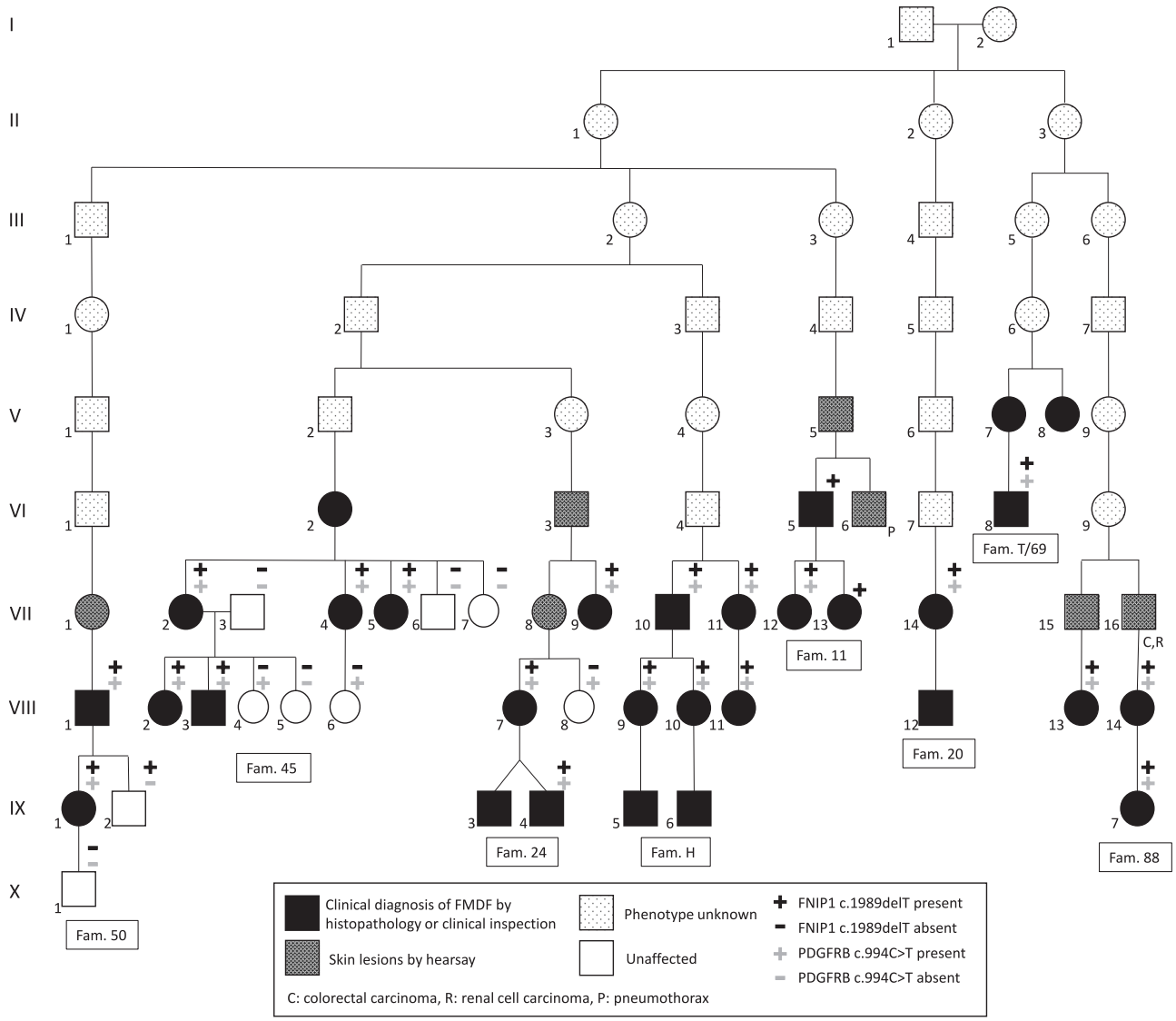

Fig. 1 Pedigree of the 8 families for which common ancestry was confirmed

Whole exome sequencing

Based on the results of recent studies, describing a *FNIP1*-associated autosomal recessive disease without a skin phenotype [28, 29], we performed more extensive genetic testing. WES was performed in patients VI-8 and VIII-10 to evaluate for other rare shared variants as the possible cause of FMDF. All rare shared variants that were identified were located close to *FNIP1* on chromosome 5. Besides the already identified variant in *FNIP1*, three rare missense variants were detected in *PCDHA10*, *PCDHA11* and *PDGFRB*. In addition, three intronic and 3'-UTR variants with an allele frequency of <1% in control databases were detected in *ARHGAP26*, *HMGXB3* and *TCF7* (Table 1). We further studied the c.994 C > T, p.(Arg332Trp) missense variant in *PDGFRB*. This variant is located most distant from *FNIP1* and is reported once in 212568 alleles in GnomAD [32]. It affects a weakly conserved amino acid that is limited to the amino acids arginine or lysine in higher organisms. The physicochemical difference between arginine and tryptophan is moderate (Grantham score 101) and the predictions on pathogenicity of the variant models give conflicting results (Align GVGD class C15; SIFT deleterious). The variant is located in the 4th Ig-like C2-type domain of the protein (UniProt). To assess whether the *PGDFRB* variant segregated with the FMDF phenotype in the other patients, we performed Sanger sequencing of the variant in patients and their relatives from whom DNA was available. The results are shown in Fig. 1. In summary, all tested patients with FMDF were found to carry the *PDGFRB* variant. The only individual with the *FNIP1* variant but no FMDF phenotype (IX-2) was not a carrier of the *PDGFRB* variant. However, three of the remaining seven unaffected individuals with an a priori risk of 50% of carrying the mutant allele who did not carry the *FNIP1* variant, were heterozygous carriers of the *PDGFRB* variant. These three individuals were adults (ages around 30, 40 and 50) at clinical evaluation. We consider them truly unaffected, since the trichodiscomas in the affected individuals were present from childhood.

Genome-wide linkage analysis

We set out to identify whether other genomic loci were linked to the FMDF phenotype. SNP genotype data was obtained from eight affected individuals (VI-8, VII-2, VII-14, VIII-1, VIII-10, VIII-11, IX-4, IX-7) and four unaffected individuals (VIII-4, VIII-6, VIII-8, IX-2). We investigated autosomal dominant inheritance for two scenarios. Scenario A included eight affected and four unaffected individuals, scenario B included only the eight affected individuals. For both scenarios, only a single locus on chromosome 5 demonstrated a maximal logarithm of the odds (LOD) score, making the

Table 1. Detected rare variants in the maximal shared haplotype

Gene	Variant	Protein	Predicted effect	Transcript	Allele frequency GnomAD	Phenotype associated with pathogenic germline variants in the gene
FNIP1	c.1989delT	p.Val663fs*	Frameshift with premature stopcodon	NM_133372.3	0	Absent B cells, agammaglobulinemia, and hypertrophic cardiomyopathy (autosomal recessive)
TCF7	c.1168 + 87 C > T		NA	NM_001346425.2	0	None
PCDHA10	c.1687G > C	p.Ala563Pro	Missense	NM_0318593	0	None
PCDHA11	c.376 A > G	p.Ile126Val	Missense	NM_018902.4	7/251,446	None
ARHGAP26	c.*30 T > A		NA	NM_015071.4	4/227,978	None
HMGXB3	c.811-20 C > T		NA	NM_014983.2	300/189,992	None
PDGFRB	c.994 C > T	p.Arg332Trp	Missense	NM_002609.3	1/217,768	Basal ganglia calcification Kosaki overgrowth syndrome Infantile myofibromatsosis Premature aging syndrome Penttinen type (autosomal dominant)

area on chromosome 5 the only linked locus genome-wide. For scenario A, the highest LOD score was 13.5 at 0.99 penetrance, which linked to the locus on chromosome 5 at 127–139 cM (Fig. 2A). In this scenario, *FNIP1* (130 cM) is within the genome-wide highest scoring locus, while *PDGFRB* (149 cM) is excluded from this locus. The highest LOD score for scenario A at 0.70 penetrance was 13.1, linking to 128–138 cM on chromosome 5 (data not shown). For scenario B, the highest LOD score was 12.07 at 0.99 penetrance (Fig. 2B) and 12.02 at 0.70 penetrance (data not shown), for a locus on chromosome 5 between 125 to 150 cM. In this scenario, all affected individuals share the variants in *FNIP1* and *PDGFRB*. The difference in the size of locus between scenarios A and B is likely the result of the inclusion or exclusion of IX-2 (unaffected) whom carries the *FNIP1* variant, but lacks the *PDGFRB* variant.

There are 268 gene located in largest locus with the highest LOD score from scenario B (125–150 cM, corresponding to 125–151 Mb), these are listed in Table S1. Among these, 107 are located in the locus with the highest LOD score from scenario A (127–139 cM). If the phenotype in the extended family is caused by a single fully penetrant variant in the identified locus, the causal variant should be located within these regions. The OMIM morbid genes from the loci and their phenotypes are listed in Table S2. None of these phenotypes resemble the phenotype in the here presented families.

DISCUSSION

We here present the presence of a shared heterozygous *FNIP1* variant in 24 patients with FMDF, of which 23 could be linked to one ancestral couple by genealogic evaluation. All affected individuals had multiple trichodiscomas, as described by Starink et al. [10]. There were no clear signs of an increased risk for BHD-associated features of lungs and kidney. Both pneumothorax (1 in 37) and renal cell carcinoma (1 in 37) occurred less frequent among the FMDF patients than would be expected based on the estimated lifetime risks for renal cell carcinoma (16%) and pneumothorax (29%) in BHD [33]. Furthermore, the only reported RCC was a papillary type 2 renal cell carcinoma, which is uncommon in BHD [34]. Therefore, the cases of renal cancer and pneumothorax most likely occurred by co-incidence in patients with FMDF. There was no informed consent for molecular studies on the renal cell carcinoma.

Initially, we concluded that the FMDF phenotype was likely to be caused by the *FNIP1* variant. However, a recent studies describing an autosomal recessive disorder caused by homozygous and compound heterozygous *FNIP1* variants without a skin phenotype in the patients and their heterozygous parents [28, 29], warranted further testing. We performed WES in two affected patients and found six more shared rare variants which were all located in proximity to the *FNIP1* gene on chromosome 5. The shared variant most distant from *FNIP1* was located in the *PDGFRB* gene. We further analyzed the presence of this variant in the other patients and unaffected family members. All affected patients were found to carry this variant, meaning that all patients with FMDF share a haplotype on chromosome 5 of at least 18.5 Mb between the rare variants identified in *FNIP1* and *PDGFRB*. Since no rare shared variants were detected with WES outside the *FNIP1* locus, we hypothesized that the *FNIP1* haplotype was the only shared haplotype between affected patients. To confirm this, genome-wide LOD scores were calculated and indeed, the haplotype on chromosome 5 was the only haplotype that reached the highest possible LOD score.

Several underlying genetic mechanisms could explain the observed phenotype in the families with FMDF

The *first hypothesis* is that the *FNIP1* variant is the cause of the FMDF phenotype. The *FNIP1* variant is a good candidate based on

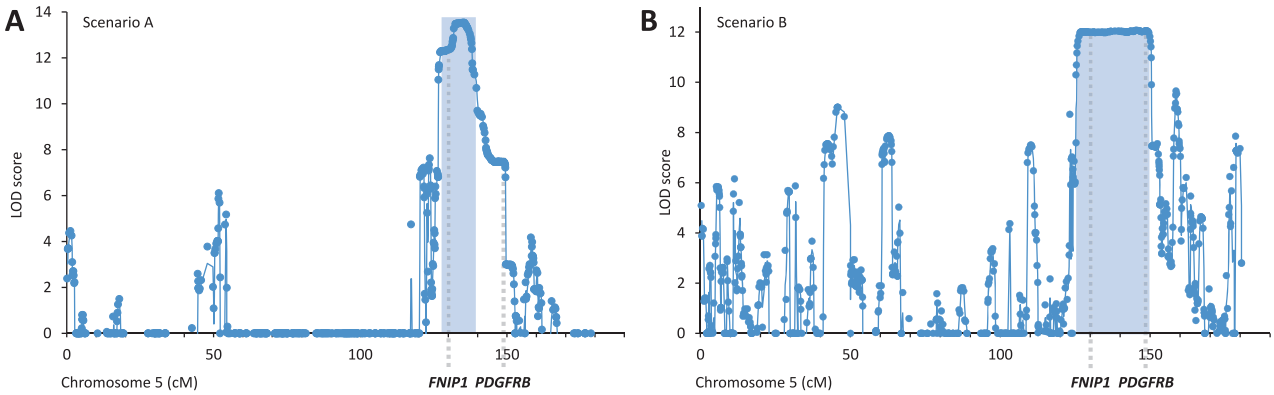

Fig. 2 Linkage analysis results of chromosome 5 showing a maximal LOD score for the locus on chromosome 5 in scenario A (**A**) and scenario B (**B**)

the fact that it is located in the only haplotype linked to the FMDF phenotype, the interaction with FLCN and the predicted effect of the variant. The detected variant is predicted to lead to a frameshift, premature stop codon and most likely nonsense-mediated decay and haploinsufficiency. We were not able to analyze whether loss of heterozygosity (LOH) for *FNIP1* was present in the trichodiscomas in the families with FMDF, since DNA extractions from FFPE material of the lesions yielded an insufficient amount of DNA and no suitable antibodies were available for immunohistochemistry. There was only one unaffected individual who carried the *FNIP1* variant who was in his 40's. This could possibly be attributed to reduced penetrance, a well-known feature of the BHD skin phenotype [5]. However, if the *FNIP1* variant is the causal variant for FMDF, it remains unclear why the patients with immunodeficiency, hypertrophic cardiomyopathy and pre-excitation syndrome and their heterozygous parents did not have a skin phenotype [28, 29]. Most of the reported *FNIP1* variants, as well as the variant that we detected, are predicted to have a similar disrupting effect on protein function, most likely by nonsense-mediated decay. The truncating variants reported in the literature are located at different positions throughout the gene, both proximal and distal to the *FNIP1* variant that is present in the FMDF families. The absence of a skin phenotype in the reported families with truncating variants in *FNIP1* could be attributed to a difference in genetic modifiers, a difference in environmental factors, or to reduced penetrance. Also, it is possible that there is a yet unexplained differential effect between truncating variants in the FMDF patients compared to the variants reported in the recessive disorder.

The *second hypothesis* is that FMDF is caused by the *FNIP1* variant in combination with a second variant within the same haplotype, for which the *PDGFRB* variant is a good candidate. Based on current insights, digenic inheritance is a rare phenomenon. The DIgenic Diseases DAtabase (DIDA) currently contains 54 digenic inherited diseases caused by 258 different digenic combinations [35]. We have not found definite cases in the literature of digenic inheritance where the two variants are located in proximity in the same haplotype. Such an inheritance has been proposed for multiple self-healing squamous epithelioma, however this has not been proven with certainty yet [36]. We considered the possibility of digenic inheritance in these families with FMDF because all patients share a rather large haplotype without recombination in 56 meioses, while recombination within the haplotype had occurred in four out of eight unaffected individuals that were available for genetic testing. We chose to further study the *PDGFRB* variant because this gene encodes a protein involved in cell proliferation, the variant is located in the coding part of the gene and it was the variant located most distant from *FNIP1*. The *PDGFRB* gene encodes the platelet-derived growth factor receptor beta (PDGFRβ). Loss of function pathogenic missense variants in *PDGFRB* cause basal ganglia calcifications [37]. The variants in basal ganglia calcifications are primarily located in the tyrosine kinase domain, but a few pathogenic loss of function variants are located in one of the extracellular IG-like domains similar to the variant identified in the FMDF patients which is located in the 4th Ig-like C2-type domain [38]. This domain is important for receptor-receptor dimerization [39–41]. Gain of function pathogenic missense variants in *PDGFRB* are involved in Penttinen-type premature aging, Kosaki overgrowth syndrome and infantile myofibromatosis [42–44]. Recently, it was proposed that these three diseases actually represent a spectrum involving myofibromas and abnormalities of vessels, brain, skin and skeleton [45]. Myofibromas can occur in the skin and originate from myofibroblasts, which are present in many organs and play a role in wound healing and fibromatosis [46]. The germline pathogenic gain of function variants in *PDGFRB* in these three diseases are located in the juxtamembrane and intracellular domains of the gene [45]. Lastly, a gain of function variant in the kinase domain of *PDGFRB* has been described as the possible cause of progressive mucinous histiocytosis in one family [47]. Further studies in a cell model and/or animal model would be necessary to explore this hypothesis and the underlying mechanism.

The *final hypothesis* is that there is one causal variant for FMDF, other than the identified variant in *FNIP1*. This could either be the identified *PDGFRB* variant, or another, yet unidentified, variant that is located in the haplotype between the variants in *FNIP1* and *PDGFRB*. Based on the finding that the *PDGFRB* variant was present in 3 unaffected individuals and was not part of the linking haplotype in scenario A, we consider the *PDGFRB* variant less likely to be causal, although reduced penetrance remains a possibility. When assuming a fully penetrant phenotype, the linkage results confine the disease locus to the region between 127–139 cM, which contains 107 genes. The only rare shared variant in this region detected with WES was an intronic variant in *TCF7*. We have not performed RNA sequencing, but there is no predicted splice effect of this variant. *TCF7* has not been associated with Mendelian disease so far. However, TCF transcription factors play an important role in canonical Wnt/β-catenin signaling, which is also regulated by *FLCN* [48, 49]. Several other genes in this region have been associated with *FLCN* or *FNIP1/2* directly or indirectly; *IRF1* is upregulated upon *FLCN* knockout, *FNIP2* interacts with *SKP1* and Lst7 (the yeast orthologue of *FLCN*) has a weak interaction with *Sec24* (*SEC24A/B* orthologue) [50–52]. Further studies are required to study whether a variant in one of these genes, that remained undetected with WES and SNP array analyses, could play a role in FMDF.

CONCLUSIONS

In summary, we show that FMDF is linked to a haplotype that includes the *FNIP1* gene in 24 patients. We detected a truncating variant in the *FNIP1* gene as the possible causal variant, but we cannot exclude that a second (genetic) factor is also needed for the phenotype or that another variant in the haplotype is causal, such as the predicted missense variant in *PDGFRB*. When evaluating patients with trichodiscomas, FMDF should be considered in the differential diagnosis and the *FNIP1* haplotype may be analyzed. In addition, these observations may provide further insight into the currently incompletely understood mechanisms that lead to the development of fibrofolliculomas and trichodiscomas.

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AUTHOR CONTRIBUTIONS

All authors have contributed sufficiently to the intellectual content of the submission. Conceived and/or designed the work that led to the submission, acquired data, and/or played an important role in interpreting the results: IvdB, IEG, MWTT, MNHL, TMS, JAB, PCJ, EH, MJBH, QDG, JJPG, AMP, PEP, MAMS, AVP, RMFW, FHM, ACH, and QW. Drafted or revised the manuscript: IvdB, IEG, MNHL, RMFW, ACH, and QW. Approved the final version: IvdB, IEG, MWTT, MNHL, TMS, JAB, PCJ, EH, MJBH, QDG, JJPG, AMP, PEP, MAMS, AVP, RMFW, FHM, ACH, and QW.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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