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ORIGINAL ARTICLE

Leiomyoma with Bizarre Nuclei: a Study of 108 Cases Focusing on Clinicopathological Features, Morphology, and Fumarate Hydratase Alterations

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Abstract

Leiomyoma with bizarre nuclei (LBN) is an uncommon variant of uterine smooth muscle neoplasm. Involvement of fumarate hydratase (*FH*) has been suggested in the pathogenesis of a subset of LBN. The goal of our study is to assess the clinicopathological, morphological, immunohistochemical and molecular findings focusing on FH in LBNs ($n = 108$) and compare it with the findings in usual leiomyomas (UL; $n = 50$) and leiomyosarcomas (LMS; $n = 42$). Immunohistochemically, loss of FH expression was found in 67/108 of LBN, 1/50 of UL and in no LMS. Class 4/5 *FH* mutations were detected in 15/53 LBN with sufficient DNA quality for molecular analysis. Pathogenic variants of the *FH* gene were detected in neither UL nor LMS. Local recurrence after surgery was present in 18/92 of LBN patients, 7 of which were histologically verified and 2 of which were found to be LBN. Our results confirmed that LBN behave in a benign fashion, although they may relapse. *FH* gene mutations were a common finding only in LBN, but not in UL and LMS. Immunohistochemistry with an antibody against FH seems to have a good sensitivity (87%) and moderate specificity (58%) with regard to predicting *FH* gene mutations and could be used as a screening method in tumors with features suggestive of FH alterations to identify patients who are at risk for the *FH* aberrations.

Keywords Leiomyoma with bizarre nuclei · Usual leiomyoma · Leiomyosarcoma · Fumarate hydratase

Introduction

Leiomyoma with bizarre nuclei (LBN) is an uncommon variant of uterine smooth muscle neoplasm. This entity was defined in 1994 and, according to Bell's criteria, is characterized by moderate-to-severe nuclear atypia, < 10 mitoses per 10 high power fields (HPF), and no tumor type (coagulative) necrosis [1]. Initially, this lesion was termed "atypical leiomyoma with low risk of recurrence", but later the terminology was changed to "atypical leiomyoma" (ALM), pleomorphic leiomyoma and symplastic leiomyoma. LBN were originally regarded as tumors with a low risk of malignant behavior, but the current opinion is that despite the possibility of recurrence, these tumors probably behave in benign fashion. However, the experience with this entity is still limited, especially in conservatively treated tumors.

According to the latest WHO classification (2014), this tumor should be termed "leiomyoma with bizarre nuclei" and the use of the term "atypical leiomyoma" is no longer recommended (the term "atypical smooth muscle tumor" is used as a synonym for smooth muscle tumors of uncertain malignant potential) [2]. However, as the bizarre nuclei do not necessarily need to be present in certain variants of this tumor, the terminology is not yet perfect. WHO classification defines this entity as a tumor containing bizarre cells (focal, multifocal or diffuse) on a background of otherwise typical leiomyoma. Prominent eosinophilic nucleoli are a common

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finding in a certain subset of these tumors. The mitotic activity is typically low, but karyorrhectic nuclei may be present, in which case they may mimic atypical mitotic figures. The cytoplasm is usually eosinophilic and can contain globular bodies, giving the cells a rhabdoid appearance. Infarct type (hyaline) necrosis can be seen, but no tumor type (coagulative) necrosis should be present.

Molecular genetic changes occurring in LBN are largely unknown. However, recent studies have shown that some of these tumors share some genetic changes with usual leiomyoma (UL) and leiomyosarcoma (LMS), including *fumarate hydratase* (*FH*) alterations or *MED12* mutations, but the frequency of these changes is different in different entities [3, 4]. Notably, FH alterations including germline and somatic *FH* mutations and an aberrant protein expression were described in a subset of LBN, but are rarely found in UL and LMS [4–10]. On the other hand, genomic deletions of the *FH* gene were described as a common finding in all leiomyocellular tumors, according to one study [3].

The *FH* gene is located on the long arm of chromosome 1 at position 43 (1q42.3-q43). The *FH* locus encodes two isoforms of fumarate hydratase: cytosolic and mitochondrial. Moreover, the Ensemble (release 94, January 2019) and the NCBI database both contain a record of fumarate hydratase pseudogene 1 (FHP1; HGNC: 39442; Entrez Gene ID: 100873790) which includes a reversely transcribed mRNA sequence of *FH* exon 7–10 (NCBI Ref Seq: NG_032302.2) and is localized on chromosome 13q14.11 (95% of homology).

FH catalyzes the reversible hydration of fumarate to L-malate. The mitochondrial isoform performs this reaction as a part of the Krebs' cycle and as such is central to aerobic respiration. The cytosolic isoform is thought to be involved in the metabolism of fumarate, which is produced in the cytosol by several reactions [11]. Patients which carry the germline homozygous *FH* mutation (FH deficiency) present with neonatal encephalopathy and rarely survive beyond early childhood. Patients with a heterozygous *FH* mutation present with variable symptoms, including HLRCC (hereditary leiomyomatosis and renal cell carcinoma).

Although the mechanism of tumorigenesis in *FH*-mutated cells remains incompletely understood, it is believed that *FH* is a tumor suppressor gene. Therefore, the inactivation of both *FH* alleles results in the reduction or complete loss of the FH's enzymatic activity, which leads to fumarate accumulation [12]. There are several proposed mechanisms by which the resulting elevated levels of fumarate may function as an oncoprotein. Elevated fumarate may be transported out of the mitochondria into the cytoplasm, where it can competitively inhibit the function of hypoxia-inducible factor (HIF) prolyl hydroxylase, resulting in HIF accumulation. With increased HIF-1α levels, HIF target genes, such as *VEGF* and *GLUT1*, are transcriptionally activated, leading to proliferation and resistance to apoptosis, providing increased vasculature and glucose transport for these glucose-dependent FH-deficient tumors. This phenomenon is called "pseudohypoxia" [12–15]. A different hypothesis to explain the role of *FH* as a tumor suppressor includes the presence of hypermutability, as a result of oxidative damage [16]. The increased level of fumarate modifies the cysteine residues in many proteins, resulting in increased protein succination and the production of S-(2-succino)-cysteine (2SC). The loss of FH's enzymatic activity is mostly demonstrated by negative IHC staining of FH and positive IHC staining of 2SC, due to its accumulation [17]. The IHC staining of FH and 2SC has been described as highly specific for the identification of loss of FH activity [17, 18].

Patients with hereditary leiomyomatosis and renal cell carcinoma (HLRCC) are at an increased risk of developing smooth muscle tumors of the uterus and skin, as well as renal tumors. HLRCC associated renal cell carcinomas are aggressive, usually papillary carcinomas, with metastatic disease in 50% of patients at the time of presentation. Efforts to reduce the morbidity and mortality of this disease through screening for disease carriers would be beneficial [16, 19]. These patients and family members would benefit from early identification and appropriate surveillance. Germline mutations of the *FH* gene are associated not only with the risk of renal cell tumors, but less frequently also with Leydig cell tumors, ovarian mucinous cystadenomas, and cerebral cavernous hemangiomas [12].

In our study we undertook a comprehensive analysis of 108 cases of LBN focusing on their clinicopathological features, morphology, and fumarate hydratase (FH) alterations on genomic DNA and protein level. The results were compared with a control cohort of 50 usual leiomyomas (UL) and 42 leiomyosarcomas (LMS).

Material and Methods

Case Selection

Formalin-fixed paraffin-embedded (FFPE) tissue blocks and patient characterization from 1998 to 2017 were obtained from the archive files and databases of the participating institutions.

The total amount of 108 cases of LBN (Table 1) was selected for analyses. The hematoxylin-eosin slides from each case were re-evaluated to confirm the original diagnosis. A cohort of 42 cases of LMS and 50 cases of UL was used as a control group. The LMS cases included 17 uterine and 25 extragenital tumors (including skin, urinary bladder, lung, oral cavity, stomach, epididymal, and orbital tumors).

In compliance with the Helsinki Declaration, the study has been approved by The Ethics Committee of General

Table 1 Clinicopathological, histological and molecular features in 108 leiomyomas with bizarre nuclei (LBN)

Characteristics [a]	LBN cases No. 108	LBN type I No. 72 (67%)	LBN type II No. 36 (33%)
Age (mean, years)	43	43	43
≤ mean	67	52	15
>mean	41	20	21
Symptomatic[a]	50/63 (79.4%)	33 (45.8%)	17 (47.2%)
Surgery[a]			
hysterectomy	60/105 (57.1%)	34 (47.2%)	26 (72.2%)
myomectomy	45/105 (42.9%)	36 (50%)	9 (25%)
Number of tumors[a]			
solitary	59/96 (61.5%)	38 (52.8%)	21 (58.3%)
multiple	37/96 (38.5%)	27 (37.5%)	10 (27.8%)
Residual/recurrence[a]	18/92 (19.6%)	13 (18.1%)	5 (13.9%)
Metastases[a]	0	0	0
Border[a]			
sharp	68/68 (100%)	41 (56.9%)	27 (75%)
infiltrating	0	0	0
Cellularity			
low	2 (2%)	1 (1.4%)	1 (2.8%)
intermediate	75 (69%)	52 (72.2%)	23 (63.9%)
high	31 (29%)	19 (26.4%)	12 (33.3%)
Density			
low	63 (58%)	44 (61.1%)	19 (52.8%)
intermediate	28 (26%)	18 (25%)	10 (27.8%)
high	17 (16%)	10 (13.9%)	7 (19.4%)
Distribution			
focal	12 (11%)	9 (12.5%)	3 (8.3%)
multifocal	60 (56%)	42 (58.3%)	18 (50%)
diffuse	36 (33%)	21 (29.2%)	15 (41.7%)
Prominent nucleoli			
Yes	57 (53%)	49 (68%)	8 (22.2%)
No	51 (47%)	23 (32%)	28 (77.8%)
Eosinophilic nuclear pseudoinclusions			
Yes	36 (33.3%)	22 (30.6%)	14 (38.9%)
No	72 (66.7%)	50 (69.4%)	22 (61.1%)
Karyorrhectic nuclei			
Yes	19 (18%)	10 (13.9%)	9 (25%)
No	89 (82%)	62 (86.1%)	27 (75%)
Mitotic count			
0/10 HPF	79 (73%)	55 (76.4%)	24 (66.6%)
1/10 HPF	20 (19%)	14 (19.4%)	6 (16.6%)
2/10 HPF	4 (4%)	2 (2.8%)	2 (5.6%)
3/10 HPF	2 (2%)	0 (0%)	2 (5.6%)
4/10 HPF	3 (3%)	1 (1.4%)	2 (5.6%)
Rhabdoid-like cells			
Yes	61 (56%)	52 (72.2%)	9 (25%)
No	47 (44%)	20 (27.8%)	27 (75%)
Staghorn vessels			
Yes	74 (69%)	51 (70.8%)	23 (63.9%)
No	34 (31%)	21 (29.2%)	13 (36.1%)
Cases with FH mutation [b]			
No. of cases with mutation / positive IHC	2/24 (8%)	1/11 (9%)	1/13 (8%)
No. of cases with mutation / negative IHC	13/29 (45%)	11/23 (48%)	2/6 (33%)

Abbreviations: [a] Data not available for all cases. [b] Mutation analysis includes 53 LBN; pathogenic, likely pathogenic and predicted pathogenic mutations (class 4/5) were considered

University Hospital in Prague. The research has also received approval from the institutional review board.

The following microscopic features of LBN were assessed: the border (sharp, infiltrating), the cellularity, the density and distribution of atypical nuclei, the type of vessels (small, staghorn, and thick and large), vascular changes (fibrinoid necrosis, luminal vascular obliteration and perivascular inflammation), the presence of hydropic change or hyalinization, and the presence of necrosis. Tumor cellularity was assessed as follows: low, moderate (approximate to the cellularity of UL), and high (approximate to the cellularity of cellular leiomyoma). The distribution of bizarre nuclei (BN)

within the LBN was assessed as: focal - if BN were only present in scattered areas in an amount arbitrarily defined as up to 5 (per ×20 objective field), multifocal - if BN were still patchy and > 5 (per ×20 objective field), and diffuse - if BN were seen essentially throughout the entire tumor. The density of BN within the LBN was semi-quantitatively assessed as follows: low: <30% of tumor cells, intermediate: ≥30 and < 70%, and high: ≥70%.

The cytologic features included: the character of chromatin, the presence of prominent eosinophilic nucleoli, karyorrhectic nuclei, nuclear pseudoinclusions, rhabdoid-like cells, and mitotic count (per 10 high power fields (HPFs)).

The LBN cases were divided into 2 subtypes, type I and II, according to their distinct nuclear features, as described by others [5]. Type I LBN were characterized by their large round or oval nuclei, distinct and smooth nuclear membranes, prominent nucleoli and open, coarse chromatin. Type II LBN were characterized by their elongated or spindled nuclei, irregular nuclear membranes, pinpoint or no nucleoli, and dark smudgy chromatin. Cases were defined as either type I or II when over 70% of the tumor cells appeared to show nuclear features which favored either one or the other.

For the tissue microarray (TMA) construction, the eligible areas of tumor were identified and marked. The tissue cores (each 2.0 mm in diameter) were drilled from different areas in a single donor block (formalin-fixed paraffin-embedded (FFPE) tissue) from each case using the tissue microarray instrument TMA Master (3DHISTECH Ltd., Budapest, Hungary). In cases of LBN samples, 2 cores were taken from areas with bizarre nuclei (arbitrary termed "R"), and 2 cores from the areas where the typical leiomyoma is on the background (arbitrary termed "G"), in order to independently evaluate the R and G areas. In 9 cases, however, only the areas with atypical nuclei were taken, as there were no areas with a typical leiomyoma appearance. In cases of UL and LMS samples, 2 cores from each biopsy were taken.

Immunohistochemical Analysis

Immunohistochemical analysis was performed manually on 4 μm sections with an antibody against FH (polyclonal, 1:500, Abcam, Cambridge, United Kingdom). The epitope retrieval solution of pH 9.0 (Leica Biosystems, Wetzlar, Germany) was used for pretreatment. The expression of FH was double-blindly evaluated by two observers. We graded the staining as negative (0) and positive (1+, 2+, 3+), on the basis of its intensity. Grade 0 signifies a complete absence of staining of the tumor cells, grade 1+ weak staining, grade 2+ moderate staining, and grade 3+ describes strong labeling. Only the cytoplasmic positivity was evaluated. In each LBN the R and G areas were analyzed separately. Overall, cases with a negative staining pattern in at least the R areas were considered as IHC negative (Table 2). Endothelial cells showing a positive staining were used as an internal control.

Molecular Analysis

Genomic DNA was isolated using the Cobas® DNA Sample Preparation Kit (Roche, Basel, Switzerland) according to the manufacturer's protocol, and then spectrophotometrically quantified using the NanoDrop 2000 instrument (Thermo Fisher Scientific Inc., Waltham, MA, USA). The quality was ascertained using a 2% agarose gel electrophoresis, and control qPCR amplification using 5× HOT FirePol EvaGreen HRM Mix NO ROX (Solis Biodyne, Tartu, Estonia). Samples with poor DNA quality (Cp > 40) in the control amplicons of sizes 237 and 308 bp were excluded from further analysis (80/200 samples). DNA sequence analysis of the *FH* gene was successfully performed in 53 LBN, 20 LMS, and 47 UL (120/200 samples).

PCR and direct Sanger sequencing of the whole coding region (10 exons) and the adjacent intronic sequences was performed. Fragments of interest were amplified using 5× HOT FirePol EvaGreen HRM Mix NO ROX (Solis Biodyne). BigDye Terminator v3.1 Ready Reaction Mix (Applied Biosystems, Thermo Fisher Scientific Inc., Waltham, MA, USA) was used for the sequencing reaction. Separation was performed on an ABI 3500 genetic analyzer (Applied Biosystems). Due to the limitations of the direct Sanger sequencing method, we were only able to detect variants with frequency over 15 or 20%, and due to the semi-quantitative manner of the method, only a rough estimation of the frequency of the mutant allele in comparison to the wild-type sequence was possible.

All samples with class 4/5 mutations or multiple low frequency variants detected by dideoxy sequencing which passed DNA quality criteria (32 cases, including 18 LBN, 6 UL, and 8 LMS) were selected and re-sequenced using next generation sequencing (NGS) in order to exclude false positive detections

Table 2 The IHC expression of FH in 108 LBN cases in separated areas with (R) or without (G) cells with bizarre nuclei

IHC staining in R and G areas	IHC evaluation	108 LBN cases
R-, G-	negative	59 (54.6%)
R-, G0	negative	5 (4.6%)
R-, G+	negative	2 (1.9%)
R+, G+	positive	38 (35.2%)
R+, G0	positive	3 (2,8%)
R-, R+, G0	negative	1 (0.9%)

Abbreviations: *LBN* leiomyoma with bizarre nuclei, *FH* fumarate hydratase, *R* areas with bizarre nuclei, *G* areas without bizarre nuclei, *G0* cases without G areas, −/+ negative/positive FH expression

of a mutation due to either fixation artifacts or existing pseudogenes. A Nimblegen custom panel sequence capture (257 kbp, 89 genes or gene parts including whole coding sequence of *FH*) of samples prepared using the Hyper Plus Library preparation kit (KAPA) was performed according to the Roche SeqCap EZ Library protocol. The samples were paired-end sequenced by MiSeq instrument (Illumina) using the 2 × 75 bp Reagent Kit v3 (Illumina). Demultiplexed FastQ files were processed using the in-house biostatistic pipeline (including PCR duplicate and low-quality reads removal), performed by the NextGENe *v*2.1.2 software (Softgenetics). The average coverage of samples in the *FH* gene was in the range of 250-500x, which shows sufficient sensitivity for the NGS detection of mutations with a frequency > 5% (at least 10 reads with mutation).

Annotation of Variants

The nomenclature of variants follows the recommendations of the Human Genome Variation Society (HGVS), and is based on the NM_000143.3 reference sequence. Mutations which were not found in the literature or databases until January 2019 were considered as novel (dpSNP, http://www.ncbi.nlm.nih.gov/SNP/; 1000 Genomes, http://www.1000genomes.org/; ClinVar, https://www.ncbi.nlm.nih.gov/clinvar/; ESP, http://varianttools.sourceforge.net/Annotation/EVS; COSMIC, http://www.sanger.ac.uk/cosmic; or HGMD, https://www.qiagenbioinformatics.com/products/human-gene-mutation-database/).

The in silico tool Variant Effect Predictor (Ensemble; http://www.ensembl.org/info/docs/tools/vep/index.html) was used to evaluate the predicted effects of all of the variants [20]. A variant was considered as a "true" pathogenic when identified as pathogenic in the mutation databases (ClinVar) or when the nature of the mutation suggests a truncated protein product (nonsense, frameshift, start loss or indels mutations). A variant was considered as a "predicted" pathogenic when at least six of the nine in silico predictive softwares suggested a damaging nature of the variant (including CADD, GERP++, Mutation Assessor, Mutation Taster, Provean, SiPhy, VEST3, PolyPhen and SIFT).

Statistical Analysis

The software STATISTICA 10 (StatSoft, Tulsa, OK, USA) was used. The Fisher exact test or the chi-square test were used to compare different tumor groups based upon the immunohistochemical and clinicopathological variables. All tests were two sided, and a p value less than 0.05 was considered significant.

Results

The patients' characteristics, clinicopathological, gross and microscopic features of LBN cohort are summarized in Table 1. In a nutshell, the mean age was 43 years (range 25–84), and the tumor sizes ranged from 5 to 150 mm. Most patients included in this study were admitted to hospital for abnormal uterine bleeding ($n = 36$; 33.3%), or pelvic pain/discomfort ($n = 9$; 8.3%). Other patients were either asymptomatic ($n = 13$; 12%) or had clinical symptoms of another nature (hydronephrosis, myomatous uterus, leiomyoma protrusion, vaginal discharge, $n = 5$; 4.6%). No data was available for 45 patients (41.7%). Of the 108 LBN, 104 (96%) were localized in the uterus, 2/108 (2%) in the parametrium and in 2 cases (2%) the location was unknown. Local recurrence after surgery was present in 19.6% (18/92) of patients – of which 11 patients presented clinically with a myomatous uterus without a bioptic confirmation of the lesions, 2 had LBN, 4 UL and the last one had mitotically active leiomyoma. Additionally, two of the patients who were treated for LBN later on developed LMS – one arising in the vaginal stump 8 months after a hysterectomy, while the second one was a case of uterine LMS occurring 7 years after a myomectomy. In both these cases the tumors arose without a relation to LBN, and as such LBN cannot be regarded as their precursor. Metastases were not observed in any of the cases. None of our patients had a personal history of renal neoplasia; although it had been reported that one patient's father died of generalization of renal clear cell carcinoma (RCC) at the age of 61.

Microscopic evaluation revealed that all LBN cases had sharp, well-circumscribed margins (68/68; 100%). However, the margins could not be ascertained in 40/108 of the LBN cases as their interface with the myometrium was not observed. A majority of cases showed both intermediate and high cellularity (106/108; 98%), and only 2/108 (2%) cases were hypocellular. Fibrinoid necrosis of the vessel wall was found in 6/108 (6%) cases, luminal vascular obliteration in 5/108 (5%), and a perivascular inflammatory infiltrate composed of mononuclear cells in 30/108 (28%). Tumor regressive changes like edema were present in 31.5% (34/108) of the cases, hyalinization in 33.3% (36/108), and hydropic changes in 3.7% (4/108). The ischemic type of necrosis or a suspicion of this type of necrosis was observed in only 4.6% (5/108) cases.

The LBN were further divided into two subgroups: type I (72 cases) and type II (36 cases) (Fig. 1a, b). The LBN of type I and II were significantly different, based upon the histopathological characteristics presented in Table 1. LBN type I more frequently had prominent nucleoli ($\chi^2 = 20.23$, df = 1, $p < 0.001$) and rhabdoid-like cells ($\chi^2 = 21.77$, df = 1, $p < 0.001$) when compared with type II. There was no difference between the presence of staghorn vessels in type I and type II lesions.

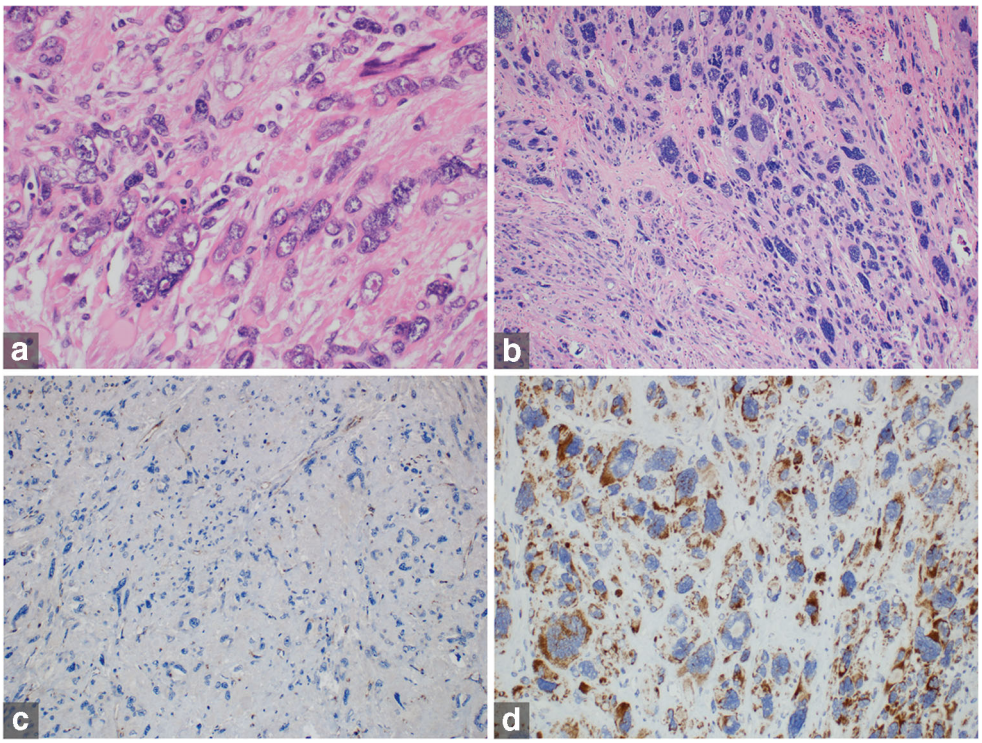

Fig. 1 Leiomyoma with bizarre nuclei. Note the prominent eosinophilic nucleoli in the type I. tumor (1A, HE, 400 x) and coarse, dark smudged chromatin in the type II. tumor (1B, HE, 100 x). Immunohistochemical expression of FH showing granular positivity in all tumor cells (1C, 200 x). Loss of FH expression in tumor cells (1D, 200 x). Note the granular positivity in endothelial cells

Immunohistochemical Findings

Immunohistochemical findings are summarized in Table 3. Briefly, the immunohistochemical analysis showed at least a partial loss of FH expression in 67/108 (62%) of LBN cases. In 97/108 (90%) cases the expression was concordant in both of the evaluated regions, R and G (Fig. 1c, d). In 8/108 (7%) cases the G area could not be evaluated due to the presence of diffuse atypias without background areas of typical leiomyoma. In 3/108 (3%) of LBN cases the noted expression was different in different areas: R versus G ($n = 2$) or in 2 different cores from the R area ($n = 1$; R-, R+, G not present) (Table 2). More cases with loss of FH expression were observed in LBN type I group in comparison to the type II group ($\chi^2 = 18.89$, df = 1, $p < 0.001$). In the group of type I LBN 76% (55/72) showed loss of FH expression, compared to 33% (12/36) in type II LBN group. In UL, loss of FH expression was present in 1/50 (2%) case. No cases of LMS showed loss of FH expression.

Molecular Findings

Sequencing analysis was performed primarily using direct Sanger sequencing, but this approach showed a high number (243) of detected *FH* variants with a relatively high percentage of C>T or G>A substitutions (97/243; 40% and 93/243; 38%), respectively, suggesting the presence of sequenced fixation artefacts (likely deamination which arose from fixation). The majority of these substitutions (82% of C>T and 62% of G>A) showed low frequency pattern with an estimated variant allele frequency of less than 25% on Sanger electropherograms. Moreover, multiple low frequency mutations were detected in numerous other samples. Therefore, the NGS capture approach was implemented to set up cut-offs to filter out the high mutation background. The NGS results were compared to Sanger sequencing data. Low-frequency variants detected using Sanger sequencing (variants the signal of which was less than one third of the wild-type signal on the electropherogram) were not confirmed using the NGS approach.

Table 3 The IHC expression of FH in different diagnoses

FH expression	LBN type I	LBN type II	LBN	UL	LMS
No. of evaluated cases	72	36	108	50	42
negative	55 (76%)	12 (33%)	67 (62%)	1 (2%)	0
positive	17 (24%)	24 (66%)	41 (38%)	49 (98%)	42 (100%)

Abbreviations: *LBN* leiomyoma with bizarre nuclei, *UL* usual leiomyoma, *LMS* leiomyosarcoma

Therefore, those variants were considered as fixation artifacts that were, together with the so-called pseudogene variants, filtered out. Two *FH* pseudogenes were identified due to the localization of some NGS reads in all of the sequenced cases outside of the coding sequence of *FH*. Firstly, an already described pseudogene *FHP1,* and secondly another copy of this pseudogene, which has not yet been described in databases. This novel pseudogene was found by comparing the sequence fragment (with the mutation identified using Sanger sequencing) to the human genome using BLAST/BLAT on chromosome 5p13.2. This copy shows 89% homology.

Altogether, DNA sequence analysis of the *FH* gene was successfully performed in 53 LBN, 20 LMS, and 47 UL cases, and revealed 57 alterations in 39/120 lesions, including 56 single nucleotide variants and one complex pathogenic variant (c.[278T>C;282del1A;284C>G], p.[Ile93Thr; Ala95ValfsTer5]). Out of these variations, 6 were protein truncating [6/57 (10.5%)], 25 missense [25/57 (43.9%)], 8 silent [8/57 (14%)] and 17 were non-coding [17/57 (29.8%)]. None of these were found in the general splice site DNA consensus motifs.

Out of all the 57 identified variants, 51 variants were unique (48 were detected once, 2 were detected in two samples, and one intronic variant was detected in 5 samples). Of the unique variants, 24 have already been previously described in databases and 27 variants were novel. Moreover, 18 nonsynonymous variants were in the catalytic domain of FH spanning codons 58–389. Pathogenic variants, according to the ClinVar database or truncating nature of the variant (excluding in silico analyses of missense variants), were detected in 7/53 (13.2%) LBN. Furthermore, 9 missense variants with an unknown significance which were determined using in silico tools as pathogenic or damaging were detected in 8/53 LBN (15.1%; 1 case carried 2 mutations).

Pathogenic or likely pathogenic mutations (class 4/5) were detected in 35% (12 out of 34) of the analyzed type I LBN and 16% (3/19) of type II LBN (summarized in Table 1). In total, 13/29 (45%) LBN with loss of FH expression, and 2/24 (8%) LBN with positive FH expression carried a class 4/5 mutation of the *FH* gene. No pathogenic *FH* gene variants were detected in UL and LMS.

In total, pathogenic, likely pathogenic, and predicted pathogenic mutations (according to databases, the truncating nature of the mutation or in silico analysis; i.e. class 4/5 mutations) were detected in 15/53 (28.3%) LBN, but in no UL or LMS cases.

The detected variants were mainly of a somatic origin. However, there was a confirmed germline pathogenic variant in exon 5 (c.698G>T, p.R233L; mutant allele frequency 50%; Fig. 2) found in one of the patients, diagnosed with LBN at the age of 36. This variant is located in the conserved sequence of the active catalytic domain (Lyase_1) of *FH*, and has been described in several publications and databases as a mutation associated with HLRCC and multiple cutaneous and uterine leiomyomas. The set of in silico prediction tools which we used in our study also suggested the damaging nature of this mutation.

Three cases of LBN showed a difference in the immunohistochemical staining of FH in an area with bizarre nuclei vs. an area with typical nuclei. In the first case (R-, G+) two likely pathogenic missense variants in exon 8 (p.G389R - mutant allele frequency 35% and p.P410L - mutant allele frequency 30%) were found. In the second case (R-, G+) and the third case (R-, R+) no mutation was found.

Discussion

Leiomyoma with bizarre nuclei (LBN) is an uncommon variant of uterine smooth muscle neoplasm. Only a few studies with at least 10 cases (either termed LBN or atypical leiomyomas (ALM)) have to date been reported, and as such less than 400 cases in total have been analyzed [3, 5, 21–28]. Additional sporadic single case reports or small series have also been reported [29].

LBN/ALM was originally regarded as a tumor with a low risk of malignant behavior, but subsequent studies have shown that this lesion probably behaves in a benign fashion. Despite this fact, LBN shares some molecular features with LMS and it has been suggested that LBN bears some potential to subsequent malignant transformation [3]. However, there are no well documented deaths related to this tumor.

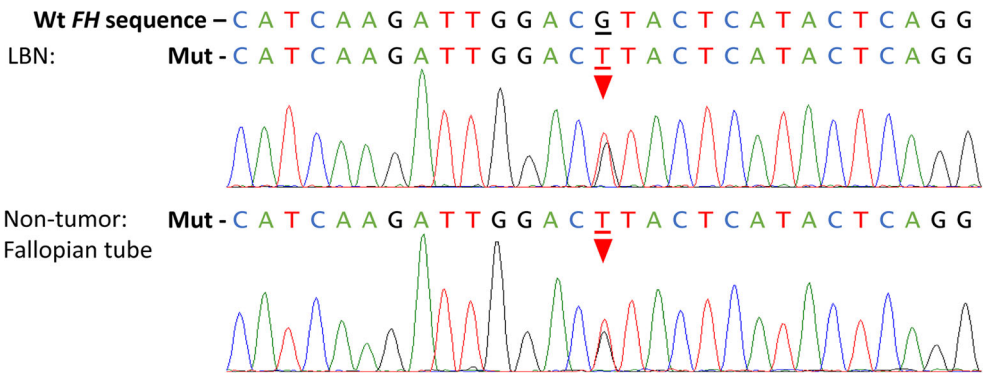

Fig. 2 Sequencing analysis revealed germline pathogenic missense mutation in exon 5 of fumarate hydratase NM_000143.3:c.698G>T; p.(Arg233Leu); rs121913123. Heterozygous variant was detected in tissue dissected from LBN, and corresponding non-tumor tissue

The optimal treatment of this tumor is still a subject with some uncertainties. Most patients are treated by hysterectomy and large studies following patients treated by myomectomy are lacking [22, 27]. In our study 19/45 (42%) patients treated by myomectomy showed clinical local recurrence. However, only 7 of these recurrences were histologically verified, of which 2 were LBN, 4 UL and 1 mitotically active leiomyoma. Based on the findings of several studies, relapse after treatment (predominantly in the form of myomectomy) was found only in the range of 0–8%, but in our study the recurrence after myomectomy was 42% according to clinical data [26, 30]. However, the LBN relapse was proven in only 2/7 histologically verified cases.

From the pathologist's point of view, LBN commonly presents a diagnostic dilemma and is probably the leiomyocellular tumor most commonly misdiagnosed as leiomyosarcoma [22]. However, when the lesion is carefully scrutinized, and the typical diagnostic criteria are ascertained, the diagnosis is usually straightforward. The biggest problem seems to be the assessment of mitotic activity, as a typical feature of these tumors is the presence of multiple karyorrhectic nuclei, which are easily misinterpreted as mitoses. In this setting, the antibody against PHH3 seems to be helpful [25, 31].

One of the other typical features of LBN/ALM is the presence of eosinophilic cytoplasmic globules. However, these are not specific for LBN/ALM and can also be found in UL, although in UL these bodies are very rare. In our previous study we were able to find them only in 10/428 usual leiomyomas (2.3%) [32]. Moreover, a staghorn pattern of vessels, nuclear pseudoinclusions, rhabdoid-like cells, and fibrillary appearance of the cytoplasm can also be found as a part of the LBN morphology.

Mutation analysis of our cohort revealed pathogenic or likely pathogenic variants of *FH* in LBN cases, but not in UL and LMS cases. Recent studies have shown that there are at least 3 putative driver mutations or cytogenetic rearrangements which occur in leiomyomas. These include the translocation t(12;14)(q15;q24), leading to an overexpression of the high mobility group AT-hook 2 (HMGA2), occurring in approximately 20% of UL. The other two mutations are the *mediator complex subunit 12* (*MED12*) mutation, occurring in 60–70% of UL, and the *FH* mutation, which can be found in approximately 20% of LBN, but is very rare in UL [5–7, 33]. In LMS, *FH* mutation and HMGA2 overexpression is rarely found [34]. However, in one study the authors found deletions of *FH* gene in 27% of patients with UL, 30.8% of patients with LBN, and 25% of patients with LMS [3].

Several studies have focused on the detailed morphological aspects of LBN/ALM and some recent studies have also focused on the molecular genetic changes which occur in these tumors [21–23]. Based on their results discussions have emerged, debating whether LBN/ALM represents a single entity or two (or more) distinct entities. In a recent study of 60 cases, the authors defined 2 subtypes of LBN/ALM based on their nuclear features [5]. Type I LBN/ALM were characterized by round or oval nuclei with smooth nuclear membranes, prominent nucleoli with perinuclear halos, and open, coarse chromatin. Type II LBN/ALM had elongated or spindled nuclei with irregular nuclear membrane, pinpoint or no nucleoli, and dark smudgy chromatin. The authors were able to classify 95% of cases, the remaining 5% showed mixed nuclear features of both types and were classified based on the predominant component. In a subsequent study (expanded to 77 cases) the authors focused on FH alterations by assessing the mutation status of the *FH* gene and immunohistochemistry with antibodies against FH and S-(2-succino)-cystein (2SC) [28]. The results of their study showed that 51% of LBN had alteration detected by immunohistochemistry and 21% of LBN harbored the *FH* gene mutation. In our study the corresponding results were 62% (67/108) and 14% (15/108), respectively. Out of the 15 cases with FH alterations, 80% were of type I and 20% were of type II LBN. We observed loss of FH expression in 76% LBN I and 33% LBN II (Table 3). Class 4/5 mutations of *FH* were more frequent in LBN I (35%, 12/34) than LBN II (16%, 3/19). The description of type I tumors in this study is quite similar to uterine smooth muscle tumors with fumarate hydratase alteration (SMT-FHs), as described recently [35–37].

In a large study of 1583 uterine smooth muscle tumors, FH deficiency was detected in 86 cases (5.43%). The frequency of FH deficiency was the highest in LBN/ALM (37.3%), while it was low in usual leiomyomas (1–2%). None of the included leiomyosarcomas showed FH deficiency. The authors detected 17 non-atypical leiomyomas with aberrant FH expression in a group of 1058 leiomyomas [38]. Morphologically, 13/17 cases contained at least some cells with mildly enlarged eosinophilic nucleoli, 13/17 with eosinophilic cytoplasmic inclusions, and 17/17 with staghorn vessels. The molecular genetic findings in 16 of these tumors showed abnormalities in the *FH* gene in 50% ($n = 8$). In this study, the authors also analyzed 182 cases of LBN/ALM. They observed loss of FH expression n 37.3% (68/182).

FH deficient tumors were often associated with *FH* mutations. These mutations vary from single nucleotide substitutions to whole gene deletions. The most commonly reported changes are single nucleotide substitutions. Among the less frequent *FH* alterations were frameshift mutations, splice site mutations, and gene deletions [7, 38]. In our study the reported variants included missense (44%) and frameshift (5%) mutations, but no splice site mutations. There is no clear evidence of a genotype-phenotype correlation. Germline mutations in the *fumarase/fumarate hydratase (FH)*-gene are associated with hereditary leiomyomatosis and renal cell cancer (HLRCC) syndrome, which predisposes carriers to the development of cutaneous and uterine smooth muscle tumors as

well as kidney cancer, frequently in the form of papillary renal cell carcinoma.

A germline missense mutation (NM_000143.3:c.698G>T; p.R233L) was determined in one patient with LBN diagnosed at the age of 36. The damaging nature of this variant is supported by several publications and database entries (ClinVar, dbSNP, HGMD) which show its association with HLRCC and multiple cutaneous and uterine leiomyomas [39, 40].

Immunohistochemistry can be helpful in the screening of tumors with suspected *FH* deficiency. *FH* is believed to be a tumor suppressor gene and inactivation of both *FH* alleles results in complete loss or reduction of the FH's enzymatic activity and leads to fumarate accumulation. This increased level of fumarate also modifies the cysteine residues in many proteins, resulting in an increased protein succination and production of S-(2-succino)-cysteine (2SC). The loss of FH enzymatic activity results in negative FH and positive 2SC on immunohistochemistry. An algorithm combining the antibody against FH and 2SC has been suggested as having a high specificity for identifying mutations in the *FH* gene, including, HLRCC-associated tumors [17, 18, 21, 37]. Tumors which are FH negative/2SC positive and/or FH positive/2SC positive are suspected of *FH* genetic alterations, including homozygous deletions and mutations [21]. The probability of *FH* mutation in tumors which are FH positive/2SC negative is very low. In one study, of the 41 2SC positive cases, 39 showed loss of FH expression. The remaining 2 cases which were positive for both FH and 2SC did not reveal any *FH* gene mutations [28]. In another study of 31 cases of LBN the authors found aberrant FH/2SC expression in 17 tumors (16 FH-negative/2SC-positive; 1FH-positive/2SC-positive). Massively parallel sequencing ($n = 24$) found that 13/14 tumors with an aberrant FH/2SC immunoprofile had *FH* gene alterations, including homozygous deletions ($n = 9$) and mutations ($n = 4$). No *FH* gene alterations were found in tumors with normal FH/2SC immunophenotype [21]. The problem of using this algorithm in routine practice is that the antibody against 2SC is, to our knowledge, currently not commercially available. Moreover, according to some studies, this antibody, despite being highly sensitive, lacks specificity. Nevertheless, the sensitivity and specificity of the antibody against FH is, according to some studies, high even when not used in combination with 2SC [8, 41].

Conclusion

In conclusion, the results of our study have confirmed that despite their worrisome nuclear features LBN behave in a benign fashion, although relapse is possible. We have found that *FH* gene mutations are a common finding in LBN, but in our study they do not occur in any of the cases of UL or LMS.

In LBN, immunohistochemistry with antibody against FH seems to have a moderate sensitivity (87%) and specificity (58%) with regard to predicting the *FH* gene mutations. We believe that immunohistochemistry can be a cheap and effective tool, which could be used as a screening method in tumors with features suggestive of FH alterations to identify patients who are at risk for the *FH* aberrations, including hereditary leiomyomatosis and renal cell carcinoma (HLRCC). However, to be able to draw definite conclusions concerning the use of anti-FH antibody in this setting future studies with larger cohorts are needed.

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Competing Interests The authors declare that they have no competing interests.

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