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ORIGINAL RESEARCH ARTICLE

Uterine leiomyomas in hereditary leiomyomatosis and renal cell cancer (HLRCC) syndrome can be identified through distinct clinical characteristics and typical morphology

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Abstract

Introduction: Hereditary leiomyomatosis and renal cell cancer (HLRCC) constitute a tumor susceptibility syndrome caused by germline mutations in the fumarate hydratase (*FH*) gene. The most common features are leiomyomas of the uterus and the skin. The syndrome includes a predisposition to early-onset, aggressive renal cell cancer. It is important to identify women with HLRCC among other uterine leiomyoma patients in order to direct them to genetic counseling and to identify other affected family members.

Material and methods: We conducted a nationwide historical study to identify typical clinical characteristics, uterine leiomyoma morphology, and immunohistochemistry for diagnosing HLRCC. The study included 20 women with a known *FH* germline mutation and 77 women with sporadic uterine leiomyomas. The patient records of all women were reviewed to obtain clinical details regarding their leiomyomas. Uterine leiomyoma tissue specimens from 43 HLRCC-related leiomyomas and 42 sporadic leiomyomas were collected and prepared for histology analysis. A morphologic description was performed on hematoxylin & eosin-stained tissue slides, and immunohistochemical analysis was carried out for CD34, Bcl-2, and p53 stainings.

Results: The women with HLRCC were diagnosed with uterine leiomyomas at a young age compared with the sporadic leiomyoma group (mean 33.8 years vs. 45.4 years, $P < 0.0001$), and their leiomyomas occurred as multiples compared with the sporadic leiomyoma group (more than four tumors 88.9% vs. 30.8%, $P < 0.0001$). Congruently, these women underwent surgical treatment at younger age compared with the sporadic leiomyoma group (mean 37.3 years vs. 48.3 years, $P < 0.0001$). HLRCC leiomyomas had denser microvasculature highlighted by CD34 immunostaining when compared with the sporadic leiomyoma group (112.6 mean count/high-power field,

Abbreviations: 2SC, S-(2-succinyl) cysteine; FH, fumarate hydratase; HIF1α, hypoxia inducible factor 1; HLRCC, hereditary leiomyomatosis and renal cell cancer; HPFs, high-power fields; IHC, immunohistochemistry; KEAP1, Kelch-like ECH-associated protein-1; SD, standard deviation; TMA, tissue microarray.

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SD 20.8 vs. 37.4 mean count/high-power field, SD 21.0 $P < 0.0001$) and stronger anti-apoptotic protein Bcl-2 immunostaining when compared with the sporadic leiomyoma group (weak 4.7%, moderate 44.2%, strong 51.2% vs. 26.2%, 52.4%, 21.4%, respectively, $P = 0.003$). No differences were observed in p53 staining.

Conclusions: Women with HLRCC may be identified through the distinct clinical characteristics: symptomatic and numerous leioymyomas at young age, and morphologic features of *FH*-mutant leiomyomas, aided by Bcl-2 and CD34 immunohistochemistry. Further, distinguishing individuals with a germline *FH* mutation enables proper genetic counseling and regular renal monitoring.

KEYWORDS
Bcl-2, CD34, fumarate hydratase, hereditary leiomyomatosis and renal cell cancer, HLRCC, uterine leiomyoma

1 | INTRODUCTION

Hereditary leiomyomatosis and renal cell cancer (HLRCC, Online Mendelian Inheritance in Man (OMIM) # 150800) constitute an autosomal dominant tumor predisposition syndrome characterized by multiple early-onset uterine leiomyomas, multiple cutaneous piloleiomyomas, and an early-onset type II papillary renal cell cancer.[1] HLRCC is caused by heterozygous germline mutations in the fumarate hydratase (*FH*) gene that inactivate the FH enzyme, causing FH deficiency and altering the function of the tricarboxylic acid cycle.[1,2] The prevalence of HLRCC is unknown, but pathogenic germline *FH* mutations have been observed in 76%–100% of families with clinical characteristics suggestive of the syndrome.[3] HLRCC-related uterine leiomyomas are highly penetrant among women who are *FH* mutation carriers (42%–100%).[4–7] The high risk for hysterectomy (53% by age 40) highlights the significant uterine disease in this tumor syndrome. Cutaneous piloleiomyomas are the most distinct characteristic (100% penetrance in men and 55% in women by age 35)[4] of the syndrome, whereas renal cell cancers (RCCs) are detected only in a subset of cases (20%–25% among *FH* mutation-positive families and 15%–21% estimated lifetime risk of mutation carrier).[7–9] HLRCC-related RCCs are exceptionally aggressive in nature, and the rate for distant metastasis is higher than in other hereditary renal cancer syndromes. Early diagnosis and clinical intervention improve the impact that uterine tumors have on health; multiple and large uterine leiomyomas deform the uterus and impair embryo implantation, causing recurrent miscarriages, preterm labor, and possibly obstruction of labor.[10] Therefore, early diagnosis is beneficial in relation to family planning.

Screening methods for identifying this inherited tumor syndrome among uterine leiomyoma patients lack accuracy. Gynecologists and pathologists play a crucial role in diagnosing the syndrome, and it is important to obtain the first intimation of HLRCC syndrome as early as possible, as the age of onset for severely symptomatic uterine leiomyomas is significantly younger compared with that for non-syndromic leiomyomas.[11] Some morphologic features of FH-deficient uterine leiomyomas have been described,[12] although these features are specific not only to syndromic *FH* mutations alone but also to HLRCC-unrelated somatic *FH* mutations.[13] Furthermore, the reproducibility and diagnostic accuracy of morphology has not reached adequate sensitivity and specificity regarding FH-mutated leiomyomas.[14] Immunohistochemistry (IHC) for FH, which manifests in FH-deficient tissue as complete loss of staining, is not recommended for excluding HLRCC, as there is evidence of retained FH staining in uterine leiomyomas with an *FH* germline mutation.[15] FH-deficient cells accumulate high levels of fumarate protein that spontaneously react with cysteine sulfhydryl groups to form S-(2-succinyl) cysteine (2SC). Succinated proteins can be detected with the 2SC antibody, and this indeed serves as a biomarker for FH-deficient uterine leiomyomas.[16] However, the 2SC antibody has only recently become commercially available, and because of the rarity of HLRCC syndrome it is very likely that most pathology laboratories do not have this antibody and staining available. Therefore, the definite diagnosis of FH-deficient leiomyomas and HLRCC syndrome is likely made in specialized centers, whereas the primary leiomyoma diagnosis and suspicion of FH-deficient leiomyomas is determined in hospitals that are less equipped for the direct or indirect analysis of mutational status.

Key message

Women with hereditary leiomyomatosis and renal cell cancer may be identified through the distinct clinical characteristics and morphologic features of FH-mutant leiomyomas, in addition to Bcl-2 and CD34 immunohistochemistry.

We conducted a nationwide study on women with HLRCC and uterine leiomyoma in order to collect a large set of tissue specimens with detailed clinical information. Our aim was to perform a histopathologic analysis with a comprehensive description of uterine leiomyoma morphology to improve the detection of HLRCC patients.

The morphologic features and the IHC evaluated in this study were selected so that they were easy to conduct in most pathology laboratories involved in basic gynecologic specimen diagnostics. Because morphologic evaluation is poorly reproducible,[14] we included IHC stainings related to previously reported findings of hypoxic and anti-apoptotic pathways in HLRCC leiomyomas and some easily recognized morphologic patterns to test if they appeared useful in differential diagnostics of HLRCC and sporadic leiomyomas. CD34 staining was selected for microvessel density evaluation. Apoptosis-related Bcl-2 and p53 stainings were selected for their easy availability and general use in most pathology laboratories. In addition, we examined the clinical characteristics of leiomyoma to further define the criteria for HLRCC syndrome. Patients meeting the proposed criteria for this tumor syndrome could then be offered genetic counseling and *FH* mutation testing to diagnose HLRCC.

2 | MATERIAL AND METHODS

2.1 | Patients and samples

All five clinical genetics departments in Finland were contacted in order to recruit women with HLRCC for our study. The departments contacted all their HLRCC patients by letter, and women with diagnosed uterine leiomyoma were asked to participate in this nationwide study.

The HLRCC uterine leiomyoma data in this study relate to 20 Caucasian Finnish patients from seven families (the data for eight of the patients had been collected previously, and we collected the data of 12 new patients who returned their informed consent to participate in the study). Hospital patient records were collected and reviewed for gynecologic patient characteristics analysis. Data on leiomyoma treatment methods were collected, and in the case of surgical treatment the pathology departments where the surgery occurred were contacted. Tissue samples, where available, were collected for histologic analyses.

The HLRCC diagnosis was confirmed from patient records on *FH* mutation testing results (Table 1). Biallelic *FH* inactivation on each uterine leiomyoma tissue specimen was determined by IHC analysis of 2SC[16] to exclude the presence of sporadic uterine leiomyomas, which are rarely observed in HLRCC patients.[17] Finally, a data set was produced of 43 HLRCC leiomyoma tissue specimens from 17 individuals.

The sporadic uterine leiomyoma group was formed from consecutive patients evaluated at Oulu University Hospital's Gynecology Outpatient Clinic. Women were chosen based on their negative family history of uterine leiomyomas and renal cell cancer (data arising from medical records and patient interviews). The group consisted of 77 women with sporadic uterine leiomyomas. Their gynecology history was reviewed carefully based on their patient records. Forty-two leiomyoma tissue specimens from 32 women were available for the histology analyses. These women had undergone either a hysterectomy or a myomectomy, and their tissue samples were available for our analyses. Again, the biallelic *FH* inactivation status was determined by 2SC IHC, which is a robust biomarker for detecting FH-deficient tumors.

2.2 | Clinical characteristics analysis

Patient records that were available were reviewed for clinical data collection. We then compared the data between 17 HLRCC and 32 sporadic cases in order to test whether there are distinct clinical characteristics that distinguish the HLRCC patients from sporadic patients.

2.3 | Screening of the *FH* status

Formalin-fixed paraffin-embedded uterine leiomyoma tissue blocks were obtained from pathology departments. Tissue microarrays (TMAs), including all collected HLRCC-related and sporadic uterine leiomyoma samples, were conducted before the analysis. To include the most representative areas of the samples in TMAs, 5-μm sections cut from all formalin-fixed paraffin-embedded tissue blocks were stained with hematoxylin & eosin, and tumor areas were marked by gynecologic pathologists (RB and AA). Four cores (diameter 0.8 mm) were punched from the original sample block and inserted in TMA paraffin blocks. Cores of normal myometrium were included in the TMAs as controls. A manual tissue arrayer (MTA-I, Beecher Instruments) was used to construct TMAs.

Non-commercial 2SC antibody, kindly provided by Norma Frizzell (University of South Carolina), for IHC was used to assess the biallelic inactivation of *FH*, as described in Kämpjärvi et al.[17] Staining was performed with an EnVision™+kit (Dako). Tissue samples were incubated with anti-2SC antibody (1:2000) at +4°C overnight, and anti-rabbit horseradish peroxidase polymer was used to detect antibody binding. Samples displaying strong nuclear and cytoplasmic staining were scored as positive (+), indicating biallelic inactivation of *FH*, and samples showing no staining or only low cytoplasmic positivity in single cells were scored as negative (−). 2SC IHC staining was assessed by a pathologist (RB).

2.4 | Morphologic and immunohistochemical analysis

Hematoxylin & eosin-stained slides from 43 uterine leiomyomas removed from 17 HLRCC patients and 42 sporadic leiomyomas from 32 patients with no evidence of HLRCC were reviewed. In addition, a set of routine diagnostic IHC stainings was used (CD34, Bcl-2, and p53). IHC was carried out using the Novocastra Novolink Polymer Detection Systems Kit (Leica Microsystems) according to the manufacturer's protocol. Primary antibodies (mouse anti-human) were

TABLE 1 Genotypic and phenotypic data on 20 hereditary leiomyomatosis and renal cell cancer (HLRCC) cases

Case	Family	*FH* mutation (nucleotide)	*FH* mutation (protein)	Age at uterine leiomyoma diagnosis	Age at uterine operation	Symptoms	Number of uterine leiomyomas*	Number of leiomyoma specimen	Diameter of largest uterine leiomyoma (mm)	Other tumors
1	FAM1	c.671_672delAG	E224fs	39	39	Menorrhagia and abdominal pain	multiple	1	30	Cutaneous piloleiomyoma
2	FAM1	c.671_672delAG	E224fs	40	40	Abdominal pain	N/A	1	N/A	Cutaneous piloleiomyoma
3	FAM1	c.671_672delAG	E224fs	48	48	Menorrhagia and abdominal pain	multiple	2	100	Cutaneous piloleiomyoma
4	FAM1	c.671_672delAG	E224fs	43	44	Abdominal pain	multiple	1	90	—
5	FAM1	c.671_672delAG	E224fs	34	35	Menorrhagia	multiple	0	45	—
6	FAM1	c.671_672delAG	E224fs	42	42	N/A	N/A	5	N/A	—
7	FAM1	c.671_672delAG	E224fs	32	39	Abdominal pain	multiple	1	110	Type II papillary renal cell tumor
8	FAM1	c.671_672delAG	E224fs	26	37	Menorrhagia and abdominal pain	multiple	1	90	—
9	FAM1	c.671_672delAG	E224fs	37	40	Menorrhagia	multiple	1	50	—
10	FAM1	c.671_672delAG	E224fs	31	38	Menorrhagia and abdominal pain	multiple	3	68	—
11	FAM2	c.583A>G	M195V	28	28	No	2	1	N/A	—
12	FAM2	c.583A>G	M195V	27	29	Menorrhagia	multiple	3	N/A	—
13	FAM2	c.583A>G	M195V	23	27	Abdominal pain	1	1	60	—
14	FAM3	N/A	N/A	46	46	Menorrhagia and abdominal pain	multiple	1	60	—
15	FAM4	c.671_672delAG	E224fs	27	42	Menorrhagia and abdominal pain	multiple	6	100	—
16	FAM4	c.671_672delAG	E224fs	37	41	Abdominal pain	multiple	7	65	—
17	FAM5	c.587A>G	H196R	28	30	Menorrhagia	multiple	7	40	—
18	FAM6	c.1027C>T	R343X	20	26	Abdominal pain	multiple	0	N/A	—
19	FAM7	c.671_672delAG	E224fs	28	34	Menorrhagia and abdominal pain	multiple	6	67	—
20	FAM7	c.671_672delAG	E224fs	40	40	Menorrhagia and abdominal pain	multiple	2	50	—

Abbreviation: N/A, data not available.

Note: Number of uterine leiomyomas was recorded as *multiple* as in the original patient documents.

used at the following dilutions: CD34 (Novocastra) 1:1000; Bcl-2 (Dako) 1:100; and p53 (Dako) 1:2400.

The IHC stainings were evaluated by two investigators (AA and OU) blinded to the mutation status. Histologic evaluation of cellularity, traditional nuclear atypia/multinucleate cells, prominent eosinophilic nucleoli with perinuclear halo, eosinophilic globules, hydropic degeneration (also described as alveolar edema), hyalinization, and mitotic activity were carried out based on the hematoxylin & eosin slides by a pathologist specialized in gynecologic pathology (AA). An evaluation of nuclear features from three areas separate from each other was conducted to ensure a relatively good degree of consistency. Eosinophilic nucleoli with a perinuclear halo were considered present if features could be observed with ×20 objective scanning.

Atypia/multinucleated cells, prominent eosinophilic nucleoli with a perinuclear halo, eosinophilic globules, hydropic degeneration, and hyalinization were reported as absent or present. Cellularity was scored as low, moderate, or high. Mitotic activity was calculated from 10 high-power fields (HPFs) of view in a hot spot. Microvessel density was defined as the number of CD34-positively stained vessels per HPF. Vessels were calculated from four HPFs in a hot spot, and average vessel density per HPF was reported. Even partial vessels seen in an HPF were included in the calculation. Four hot spot HPFs were selected so that the same vessels were not calculated more than once, meaning that the HPFs were not in close contact with each other. Nuclear and cytoplasmic Bcl-2 staining reactions were divided into four categories: 0, negative immunostaining; 1, weak immunostaining or less than 10% of cells showing positivity; 2, moderate immunostaining or 10%–70% cells showing positivity; or 3, strong immunostaining or more than 70% of cells showing positivity. The p53 results were categorized as 0, totally negative immunostaining (aberrant); 1, weak or moderate nuclear immunostaining in less than 75% of cells (wild-type); or 2, strong nuclear immunostaining in at least 75% of cells (aberrant).

2.5 | Statistical analyses

Statistical analyses of clinical characteristics (at a woman-level), morphology, and IHC (at a tissue-level) were performed using SPSS for Windows 25.0.0.0 (IBM Corp.). The statistical significance of differences between HLRCC and sporadic cases for continuous data were evaluated with independent samples *t* tests for normally distributed data and Mann–Whitney *U* tests for skewed data. Sensitivity analysis for clinical characteristics was conducted among those cases with uterine leiomyoma tissue specimens available for this study. Associations between the type of uterine leiomyoma and IHC markers and other categorical data were evaluated using cross-tabulation and Pearson chi-squared and Fisher's exact tests. A two-sided *P* value less than 0.05 was considered statistically significant. All values given are frequencies or means/medians ±standard deviation (SD).

2.6 | Ethical approval

Appropriate research permissions were obtained from the local ethics committee (Ethics Committee of the Northern Ostrobothnia Hospital District, date of approval June 15, 2009, reference number 71/2009). Informed consent was obtained from all patients taking part in the study, and in the case of deceased patients individual permission was obtained from the appropriate authorities.

3 | RESULTS

3.1 | Clinical characteristics

Women with HLRCC-related uterine leiomyomas differ significantly when comparing their gynecologic clinical characteristics to those of women with sporadic uterine leiomyomas. In this study, the women with HLRCC were significantly younger when diagnosed with leiomyomas compared with the sporadic leiomyoma group (33.8 years old vs. 45.4 years old, $P < 0.0001$). They were more frequently symptomatic at the time of diagnosis compared with the sporadic leiomyoma group (95% vs. 6.5%, $P < 0.0001$), with menorrhagia (60% vs. 3.9%, $P < 0.0001$) and lower abdominal pain (70% vs. 2.6%, $P < 0.0001$; Table 2). Definitive treatment was required at a significantly younger age compared with the sporadic leiomyoma group (37.3 years vs. 48.3 years, $P < 0.0001$; Table 2). Women with HLRCC had numerous leiomyomas more often than women with sporadic leiomyomas (88.9% having more than four tumors vs. 30.8%, $P < 0.0001$). On average, HLRCC uteri were heavier at the time of surgery (438 g vs. 367 g, $P = 0.6$) and the diameter of the largest leiomyoma was greater (68 mm vs. 56 mm, $P = 0.08$) in the HLRCC group compared with the sporadic leiomyoma group, but the differences were not statistically significant. There were no differences in fertility characteristics among the studied groups (pregnancies 2.4 vs. 2.0, $P = 0.31$; deliveries 2.0 vs. 1.7, $P = 0.33$; Table 2).

3.2 | Morphologic and immunohistochemical features

All 43 HLRCC-related uterine leiomyomas scored positive (+) on 2SC staining, and all 42 sporadic leiomyomas scored negative (–), which confirmed the *FH* inactivation status within the studied groups. All uterine leiomyomas were histologically benign. The HLRCC-related leiomyomas and sporadic leiomyomas had similar frequencies for cellularity, mitotic activity (all being typical), hydropic degeneration, and necrosis (Table 3). The HLRCC-related leiomyomas were distinguished from sporadic leiomyomas based on nuclear atypia (present in 30.2% vs. 0, $P < 0.0001$), prominent eosinophilic nucleoli with a perinuclear halo (39.5% vs. 2.4%, $P < 0.0001$), eosinophilic globules (48.8% vs. 0, $P < 0.0001$), and the absence of hyalinization (2.3% vs. 33.3%, $P < 0.0001$; Table 3, Figure 1).

TABLE 2 Comparison of clinical characteristics between hereditary leiomyomatosis and renal cell cancer (HLRCC) and sporadic uterine leiomyoma cases

	HLRCC	*N*	Sporadic	*N*	*P*	HLRCC	*N*	Sporadic	*N*	*P*
	All cases					**Cases with uterine leiomyoma tissue specimen**				
Year of birth, mean ± SD	1956 ± 12.3	20	1953 ± 6.8	77	0.11	1956 ± 12.2	17	1953 ± 6.7	32	0.39
Pregnancies, mean ± SD	2.4 ± 1.5	20	2.0 ± 1.5	77	0.31	2.5 ± 1.5	17	2.3 ± 1.4	31	0.65
Deliveries, mean ± SD	2.0 ± 1.3	20	1.7 ± 1.4	77	0.33	2.1 ± 1.3	17	1.9 ± 1.3	31	0.69
Age at diagnosis (years), mean ± SD	33.8 ± 8.0	20	45.4 ± 7.9	77	<0.0001	35.2 ± 7.3	17	44.2 ± 8.1	32	<0.0001
Symptoms at diagnosis, *n* (%)	19 (95.0)	20	5 (6.5)	77	<0.0001	16 (94.1)	17	2 (6.3)	32	<0.0001
Menorrhagia, *n* (%)	12 (60.0)	20	3 (3.9)	77	<0.0001	11 (68.8)	16	1 (3.1)	32	<0.0001
Pain in lower abdomen, *n* (%)	14 (70.0)	20	2 (2.6)	77	<0.0001	12 (75.0)	16	1 (3.1)	32	<0.0001
Surgical treatment, *n* (%)	20 (100)	20	51 (68.0)	75	0.003	17 (100)	17	32 (100)	32	
Age at surgery (years), mean ± SD	37.3 ± 6.4	20	48.3 ± 4.8	75	<0.0001	38.7 ± 5.6	17	45.9 ± 6.9	32	0.001
Uterine weight (g), mean ± SD	438 ± 315	8	367 ± 328	24	0.60	546 ± 312	7	424 ± 250	11	0.38
Number of leiomyomas, *n* (%)		18		65	<0.0001		15		27	0.001
1	1 (5.6)		28 (43.1)			0		12 (44.4)		
2–4	1 (5.6)		17 (26.2)			1 (6.7)		6 (22.2)		
>4	16 (88.9)		20 (30.8)			14 (93.3)		9 (33.3)		
Diameter of largest leiomyoma (mm), mean ± SD	65.0 ± 24.4	15	50.0 ± 31.7	69	0.08	70.8 ± 25.2	13	54.6 ± 27.5	28	0.08

Note: Sensitivity analysis was conducted among those with uterine leiomyoma tissue specimen available for this study.

TABLE 3 Morphologic features of 43 hereditary leiomyomatosis and renal cell cancer (HLRCC) related uterine leiomyoma tissue specimens from 17 cases and 42 sporadic uterine leiomyoma tissue specimens from 32 cases

	HLRCC uterine leiomyoma (*N* = 43)	Sporadic uterine leiomyoma (*N* = 42)	*P*
Cellularity, *n* (%)			
Low	0	0	
Moderate	38 (88.4%)	34 (81.0%)	
High	5 (11.6%)	8 (19.0%)	0.382
Mitotic activity, mean (SD)	0.74 ± 1.2	0.38 ± 0.8	0.112
Nuclear atypia/multinucleate cells, *n* (%)	13 (30.2%)	0	<0.0001
Prominent eosinophilic nucleoli with perinuclear halo, *n* (%)	17 (39.5%)	1 (2.4%)	<0.0001
Hyalinization, *n* (%)	1 (2.3%)	14 (33.3%)	<0.0001
Hydropic degeneration, *n* (%)	22 (51.2%)	19 (45.2%)	0.666
Eosinophilic globules, *n* (%)	21 (48.8%)	0	<0.0001
Necrosis, *n* (%)	0	1 (2.4%)	0.494

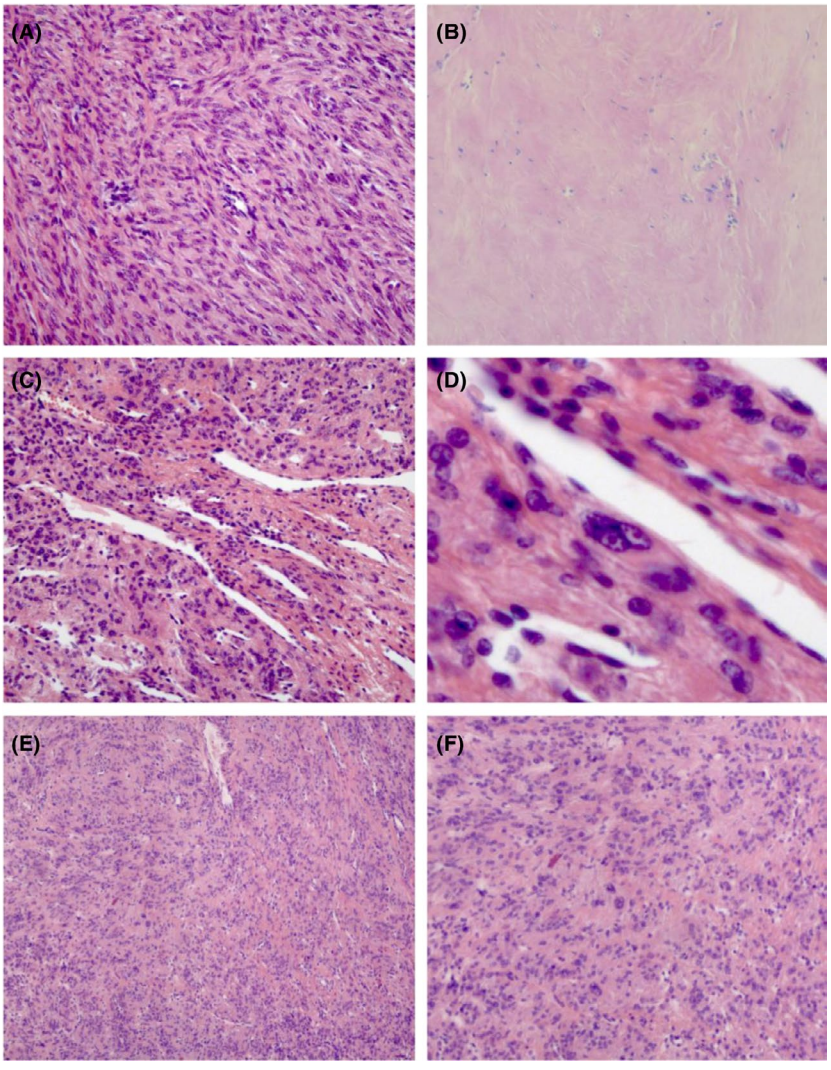

FIGURE 1 Morphological features of sporadic leiomyomas (A,B) and HLRCC leiomyomas (C–F). (A) Typical morphology, (B) hyalinization, (C) staghorn-type vasculature, (D) eosinophilic nucleoli with perinuclear halo, (E,F) multinucleated cells

HLRCC-related uterine leiomyomas showed significantly higher microvessel density than sporadic leiomyomas (112.6 mean count/HPF, SD 20.8 vs. 37.4 mean count/HPF, SD 21.0, $P < 0.0001$; Figure 2). The vessels in HLRCC myomas were partly dilated and sometimes staghorn-like (Figure 1). Anti-apoptotic protein Bcl-2 staining resulted in more frequent positivity in HLRCC leiomyomas

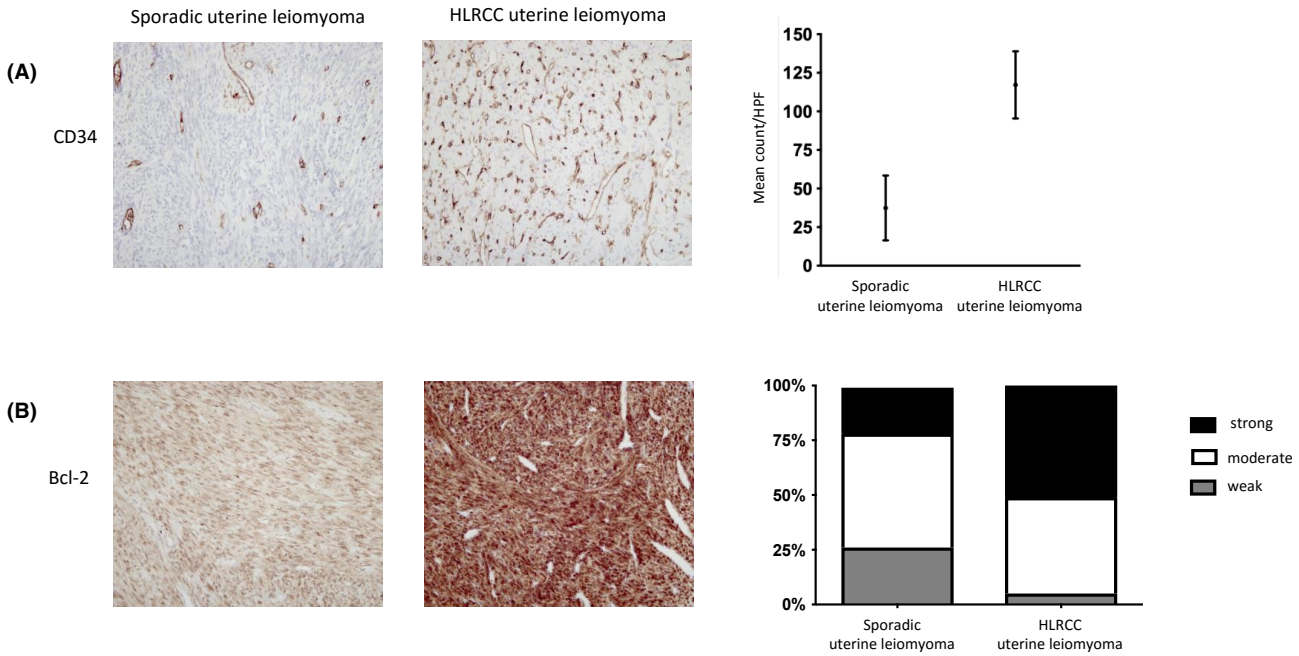

FIGURE 2 Results of the immunohistochemical stainings on sporadic and HLRCC uterine leiomyomas. (A) Microvessel density expressed through CD34 antibody staining resulted in higher mean count per high-power field (HPF) for HLRCC uterine leiomyomas when compared with sporadic uterine leiomyomas (mean ± SD: 112.6 ± 20.8 vs. 37.4 ± 21.0, $P < 0.0001$). (B) Antiapoptotic protein Bcl-2 staining (weak immunostaining <10% of cells, moderate 10%–70%, strong >70%) resulted in differing proportion profiles for HLRCC leiomyomas showing more frequent antiapoptotic cell positivity (4.7%/44.2%/51.2% vs. 26.2%/52.4%/21.4%, weak/moderate/strong, respectively, $P = 0.003$)

compared with sporadic leiomyomas (4.7%/44.2%/51.2% vs. 26.2%/52.4%/21.4%, weak/moderate/strong, respectively, $P = 0.003$) (Figure 1). All HLRCC and sporadic leiomyoma specimens displayed weak or moderate (less than 75% of cells) discontinuous immunostaining for p53, referring to wild-type mutational status.

4 | DISCUSSION

Our results suggest that suspected HLRCC-related leiomyomas can be distinguished through distinct clinical characteristics and histologic features. Women affected by this tumor syndrome have a high risk of developing uterine leiomyomas that grow in multiple numbers, so deforming the uterus considerably. Leiomyomas are highly symptomatic, and patients require surgical treatment at a young age. In histopathology analysis, these leiomyomas share morphologic features such as multinucleate cells, prominent eosinophilic nucleoli with a perinuclear halo, eosinophilic globules, staghorn-like vessels, and (our novel finding) the absence of hyalinization. IHC for CD34 and Bcl-2 could be used to further strengthen the suspicion of the syndrome. If these criteria are met, a referral to genetic counseling and HLRCC (*FH* mutation) testing is recommended.

Previously published studies have demonstrated women with HLRCC to have numerous (from 1 to 20) and large (up to 10 cm in diameter) uterine leiomyomas. When the initial diagnosis on sporadic leiomyomas is often set with incidental findings, HLRCC-related leiomyomas are diagnosed with severe symptoms, and approximately 10 years earlier (30 years vs. 40 years). Due to the enlarged and deformed uterus, these women require surgical treatment (myomectomy or hysterectomy) at young age, many times before 30 years.[3] Our results support these clinical characteristics, even though the described differences were not as great, possibly because of sample size.

HLRCC-related uterine leiomyomas have distinct tumor morphology based on (1) increased cellularity, (2) nuclear atypia/multinucleate cells, and (3) prominent eosinophilic nucleoli with a perinuclear halo, (4) eosinophilic cytoplasmic globules, (5) alveolar edema/hydropic degeneration, and (6) staghorn-like vessels.[12,15,18] Schwannoma-like nuclear palisading structures have been reported.[19] Our study does not confirm increased cell density or alveolar edema/hydropic degeneration as typical features of HLRCC-related leiomyomas. In addition, nuclear palisading was not a prominent feature in any of the leiomyomas. The findings of nuclear atypia, prominent eosinophilic nucleoli, staghorn-like vessels, and the presence of cytoplasmic eosinophilic globules are in agreement with the previously published morphologic features. The HLRCC leiomyomas with atypical nuclei in this study could fall into the WHO subgroup of "leiomyoma with bizarre nuclei" even though the amount of bizarre cells was not very abundant. We also add the novel finding of the absence of hyalinization (tissue degeneration into a translucent glass-like substance) as a distinct difference. Due to a recent blinded control-cohort study that concluded the morphologic criteria were largely irreproducible

among pathologists,[14] we added in our study a few IHC stainings to find out whether combined morphologic and IHC analysis could improve the identification of HLRCC uterine leiomyomas for further testing.

One of the proposed molecular mechanisms of HLRCC-related tumors is the so-called "pseudohypoxia" pathway, resulting from the induction of the hypoxia inducible factor 1 (HIF1α) and overexpression of the hypoxia/angiogenesis pathway genes under normoxic conditions. This is claimed to be a direct consequence of *FH* inactivation.[20] HIF1α levels are observed to be higher in HLRCC uterine leiomyomas than in the surrounding myometrium, and the expression levels of HIF1α target genes are higher in HLRCC leiomyomas than in sporadic leiomyomas.[21] In addition, microvessel density is higher in HLRCC leiomyomas than in the surrounding myometrium, whereas sporadic leiomyomas are less vascular than normal myometrium.[22] Analysis of vascular density can therefore help in distinguishing HLRCC uterine leiomyomas from sporadic leiomyomas, and microvessel density can be highlighted by an endothelial marker such as CD34 staining.

Cells placed under hypoxic conditions eventually undergo several biologic responses, apoptosis being one.[23] However, *FH*-inactivated cells overcome the apoptotic signaling independent of the HIF mechanism by activation of the energy sensor AMP-activated protein kinase.[24] Increased resistance to apoptosis was first observed in a study by Wortham et al,[25] who observed significant differences in uterine leiomyoma tissues in the expression of several anti-apoptotic proteins (Bcl-2, PCNA, Bcl-x, and Bak), suggesting stronger signals for survival and against apoptosis. Another observation regarding renal cyst formation in a mouse model is that HIF pathways do not seem to contribute to cyst formation, but fumarate accumulation leads to activation of antioxidant response pathways by Kelch-like ECH-associated protein-1 (KEAP1) succination.[26] KEAP1 is an oxidative stress sensor, and its succination results in stabilizing nuclear factor erythoid-2-related factor 2, which further induces the antioxidant response pathway promoting tumor growth. Bcl-2 is a substrate of KEAP1. Oxidative stresses induce the dissociation of Bcl-2 from KEAP1, resulting in an increase in Bcl-2:Bax heterodimers, which then reduces apoptosis and enhances cell survival.[27] We tested the usability of Bcl-2 as part of screening IHC stainings for uterine leiomyomas. Our results confirm the previous findings, as 51.2% of HLRCC-related leiomyoma tissue samples showed strong staining for Bcl-2 (>70% cells showing positivity).

The 2SC antibody used in this study to detect FH inactivation has recently become commercially available and will be a powerful tool for detecting FH-deficient leiomyomas, and consequently HLRCC.[17] However, although the 2SC antibody seems to be a specific and sensitive marker for FH-deficient leiomyomas, its use may in practice be limited to specialized centers because of the rarity of HLRCC syndrome. Commonly available routine methods could be used as described here to identify patients for in-depth HLRCC diagnostics.

This study has some limitations. HLRCC patients were recruited based on their personal information regarding a previous uterine leiomyoma diagnosis. Upon recruitment, appropriate information was given regarding leiomyoma tissue collection and the requirement of previous surgical treatment and available tissue samples for the study. The selected recruitment method might exclude women with either asymptomatic or only mildly symptomatic leiomyomas and those who had not required surgical treatment. Therefore, only women with the most severe clinical characteristics might be included in this study, enhancing the differences in clinical characteristics compared with women with sporadic leiomyomas. However, the proposed management is only applicable in terms of the histologic analysis part for those patients who do require surgery and whose leiomyoma tissue will be available for histologic analysis.

The strengths of this study are the relatively large data set for this rare syndrome and the sporadic uterine leiomyoma data set of comparable size. In addition, the patient records are well maintained and consistent at all hospitals so that information can be reliably compared. Finally, all leiomyomas were tested for *FH* inactivation status to ensure that no sporadic leiomyomas were included in the test group and no FH-deficient leiomyomas would be in the sporadic group.

5 | CONCLUSION

HLRCC-related leiomyomas have been shown to have fundamentally different tumorigenesis pathways[25] and clearly different expression profiles[28] compared with sporadic uterine leiomyomas. As HLRCC patients have an increased risk of aggressive RCC, and numerous uterine leiomyomas at a young age may affect family planning, it is important to identify these individuals. Accurate screening methods applicable to population-level routine clinical practice have been lacking for diagnosing HLRCC. Currently, patients are referred for genetic counseling based on clinicians' alertness and awareness of the syndrome. Our results suggest that screening could be enhanced through the evaluation of clinical characteristics: symptomatic and numerous leioymyomas at young age, and careful morphologic analysis, in addition to IHC of CD34 and Bcl-2.

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CONFLICT OF INTEREST

None.

AUTHOR CONTRIBUTIONS

OU and AA contributed to study planning, data collection, sample handling, data analysis and interpretation, and manuscript drafting. KK and PV contributed to data collection, sample handling, and data analysis and interpretation. RB contributed to data collection,

and sample handling. IYJ and MR contributed to data analysis and interpretation. LAA contributed to data collection, and data analysis and interpretation. OK contributed to study planning, data collection, and data analysis and interpretation. All authors contributed to manuscript revision and writing.

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