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Comparison of 2SC, AKR1B10, and FH antibodies as potential biomarkers for FH-deficient uterine leiomyomas

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Abstract

Hereditary leiomyomatosis and renal cell cancer (HLRCC) is a tumor predisposition syndrome caused by germline *FH* mutations and characterized by uterine and cutaneous leiomyomas and renal cell cancer. Currently, there is no generally approved method to differentiate FH-deficient uterine leiomyomas from other leiomyomas. Here, we analyzed three antibodies (2SC, AKR1B10, FH) as potential biomarkers. The study consisted of two sample series. The first series included 155 formalin-fixed paraffin-embedded (FFPE) uterine leiomyomas, of which 90 were from HLRCC patients and 65 were sporadic. The second series included 1590 unselected fresh frozen leiomyomas. Twenty-seven tumors were from known HLRCC patients, while the *FH* status for the remaining 1563 tumors has been determined by copy number analysis and Sanger sequencing revealing 45 tumors with mono- (n=33) or biallelic (n=12) *FH* loss. Altogether 197 samples were included in immunohistochemical analyses: all 155 samples from series one and 42 available corresponding FFPE samples from series two (15 tumors with mono- and seven with biallelic *FH* loss, 20 with no *FH* deletion). Results show that 2SC performed best with 100% sensitivity and specificity. Scoring was straightforward with unambiguously positive or negative results. AKR1B10 identified most tumors accurately with 100% sensitivity and 99% specificity. FH was 100% specific but showed slightly reduced 91% sensitivity. Both FH and AKR1B10 displayed also intermediate staining intensities. We suggest that when patient's medical history and/or histopathological tumor characteristics indicate potential FH-deficiency, the tumor's FH status is determined by 2SC staining. When aberrant staining is observed, the patient can be directed to genetic counseling and mutation screening.

Key words: Uterine leiomyoma, HLRCC, FH, AKR1B10, 2SC

Introduction

Uterine leiomyomas, or fibroids, are the most common gynecological tumors among women during reproductive years. They can be found in over 70% of women, with symptomatic lesions reported in 25–50% of cases.[1] Typical symptoms include heavy and prolonged menstrual bleeding, iron-deficiency anemia, pain and pressure in the pelvic area, and reproductive dysfunction. Economic burden caused by uterine leiomyomas (direct health care costs for leiomyoma and fertility treatments, sick leaves, increased use of sanitary products) is substantial.[2] Majority of leiomyomas display conventional histopathology, whereas 10% belong to one of the rarer variant subtypes. These variants include cellular and mitotically active leiomyomas and tumors with bizarre nuclei.[3]

Uterine leiomyomas can be divided into at least four distinct subclasses based on recurrent genetic aberrations and unique gene expression profiles.[4,5] The most common genetic alterations are specific mutations in mediator complex subunit 12 (*MED12)*, which underlie 53–75% of tumors.[6] Aberrations affecting high mobility group AT-hook 2 (*HMGA2*) and leading to significant HMGA2 overexpression are found in 10–20% of uterine leiomyomas.[7] The third well-established subgroup consists of fumarate hydratase (*FH*) -deficient tumors. Although these tumors account for only ~1–2% of all uterine leiomyomas, they form a clinically relevant subgroup as they often occur in the context of hereditary leiomyomatosis and renal cell cancer (HLRCC) syndrome.[8] Based on our recent findings, the fourth subclass carries inactivating mutations in genes encoding for members in the SRCAP-protein complex, which regulates chromatin structure and influences transcription[5].

HLRCC is a tumor predisposition syndrome characterized by uterine and cutaneous leiomyomas and aggressive type 2 papillary renal cell carcinomas.[9,10] HLRCC is inherited in an autosomal dominant manner, where one defective *FH* allele is inherited and the other is lost by a somatic mutation.[8] The age of HLRCC diagnosis is around 30 years, and the penetrance increases with age.[11,12] HLRCC-associated uterine leiomyomas are typically multiple, severely symptomatic, highly recurrent, and

often require surgical treatment.[12,13] HLRCC syndrome has been diagnosed even in the absence of cutaneous leiomyomas or renal cell cancer, and thus, the possibility of the syndrome should be considered in young patients with multiple symptomatic uterine leiomyomas or a strong family history of the disease.[14] Fumarase-deficient uterine leiomyomas typically display distinctive morphologic features.[15-17] While these characteristic traits are suggestive of FH-deficiency, they are not sufficient for diagnostic purposes. Renal cell cancers occur in a subset of HLRCC patients and tend to present later, but they often show an aggressive clinical course[11] further emphasizing the importance of early diagnosis and regular follow-up of *FH* mutation carriers.

Currently, there is no established biomarker that could be used at the clinical setting to differentiate FH-deficient tumors from other uterine leiomyomas. Direct staining with an FH antibody can be used, but it may lead to a false negative result as it also detects stable albeit non-functional protein products.[17-20] An indirect method for identifying FH-deficient tumors is immunohistochemistry with the S-(2-succino)-cysteine (2SC) -antibody, which detects succinated proteins caused by the accumulation of fumarate.[21] This has provided robust results, but the antibody has only recently become commercially available and thus has not been available for clinical use. The third potential biomarker is an antibody against aldo-keto reductase family 1, member B10 (AKR1B10). We have recently shown that at the RNA level *AKR1B10* is the most significantly overexpressed gene in leiomyomas with biallelic *FH* inactivation compared to other leiomyomas or normal myometrium.[4] The eligibility of AKR1B10 antibody in detecting FH-deficient uterine leiomyomas has not been previously studied. Here, we tested 2SC, AKR1B10, and FH antibodies as potential biomarkers for detecting FH-deficient tumors using a unique series of HLRCC-associated and unselected uterine leiomyomas.

Materials and Methods

Study subjects

The first sample series (series 1) consists of 155 FFPE uterine leiomyomas originally collected for diagnostic purposes.[22] This series includes 90 samples from 25 HLRCC patients (88 FH-deficient and two sporadic *MED12* positive tumors) and 65 sporadic FH-proficient tumors. The second series (series 2) consists of 1590 fresh frozen uterine leiomyomas and the respective normal myometrium tissue from 515 patients.[5,23-25] It includes a large collection of sporadic uterine leiomyomas (n=1563) from 512 hysterectomy patients and 27 tumors from three known HLRCC-patients. The *FH* status of these 27 tumors is known; 20 are HLRCC-associated tumors with biallelic *FH* inactivation and seven are sporadic tumors with a germline *FH* mutation and a somatic *MED12* mutation.[22] See **Supplemental Table 1** for additional information on HLRCC patients´ tumors in series 2 (Supplemental Digital Content 1).

The study has been approved by the appropriate ethics review board of the Hospital District of Helsinki and Uusimaa (HUS; 133/E8/03, 88/13/03/03/2015, 177/13/03/03/2016), Helsinki, Finland, and conducted in accordance with the Declaration of Helsinki. Archival FFPE samples were obtained with permission from National Supervisory Authority for Welfare and Health (Valvira). Fresh frozen samples in series 2 were collected with a written informed consent (449 patients) or by authorization from the director of the health-care unit (an anonymous series of 66 patients).

Defining the *FH* status for unselected uterine leiomyomas

The *FH* status of unselected sporadic uterine leiomyomas in series 2 (n=1563) has been previously determined[5], but the results have not been described in detail except for three *FH*-deficient tumors (somatic point mutation and a deletion in the other allele in tumors M4m3, M32m1 and My6213m1 reported in [26,27]). In brief, copy number analysis was performed to detect somatic deletions affecting the *FH* locus (1q43). High-throughput SNP array data (Illumina HiScan system utilizing Infinium® HumanCore-24 BeadChip Kit, Illumina Inc., San Diego, CA, USA with 4000 additional custom markers) was produced at The Estonian Genome Center, University of Tartu, Estonia. Preprocessing

of the SNP array data was done with Illumina Genome Studio version 2011.1 with default parameters. Raw copy number calls were constructed, log2-transformed, and corrected for GC waves using Partek Genomic Suite 6.6 (Partek Inc., St. Louis, MO, USA). Every uterine leiomyoma sample was compared to its respective normal myometrium to detect somatic copy number changes. To identify chromosomal regions with somatic copy number aberrations, Partek's segmentation algorithm was utilized with a minimum of 100 markers, a signal-to-noise ratio of 0.3, and a segmentation p-value of 0.001. Segments below -0.1 (deletions) were considered valid. All results were visualized in Partek to exclude technical artifacts.

When a monoallelic *FH* deletion was identified in the copy number analysis, all coding exons of *FH* were sequenced to detect potential inactivating mutations in the other allele. Sequencing was performed with ABI3730xl DNA Analyzer utilizing BigDye® Terminator v3.1 Kit chemistry (Thermo Fisher Scientific) at the Sequencing Unit of the Institute for Molecular Medicine Finland (FIMM) Technology Center, University of Helsinki. Sequencing electropherograms were analyzed utilizing Mutation Surveyor (SoftGenetics, State College, PA, USA). When a mutation was identified, the corresponding normal tissue sample was sequenced to determine whether the mutation is somatic or in the germline. Functional effect of mutations was predicted *in silico* with Combined Annotation-Dependent Depletion (CADD) and pathogenicity scores included in Varsome.[28,29]

cDNA sequencing was performed to evaluate the potential effect of two synonymous changes on splicing. Total RNA was extracted with NucleoSpin® RNA Set for NucleoZOL (Macherey-Nagel, Düren, Germany). RNA was converted to cDNA with M-MLV Reverse Transcriptase (Promega, Madison, WI, USA). Sequencing was performed at FIMM.

Immunohistochemistry

Tissue samples in immunohistochemical analyses

All 155 samples from series 1 entered immunohistochemistochemical analyses. Tissue microarrays (TMA) of the samples have been previously constructed.[22] They include four tissue punches from each tumor and four punches from random myometrium samples, which serve as normal tissue controls. From series 2, corresponding FFPE samples were available from 15 tumors with monoallelic (i.e. one copy of the gene) and seven with biallelic (i.e. both copies of the gene) *FH* loss. These were included in the immunohistochemical analyses together with 20 tumors with no *FH* deletion, which served as controls. This totals 42 sporadic tumors from series 2. Two FFPE blocks that were available from the one HLRCC patient in series 2 have been included in the TMA of series 1 and analyzed as part of that cohort. With both series 1 and series 2 samples combined, a total of 197 uterine leiomyoma samples entered immunohistochemical analyses.

Immunohistochemical staining

For immunohistochemistry, 5µm tissue sections were deparaffinated with xylene. Heat-induced antigen retrieval was performed by heating the samples in citrate buffer (pH 6.0) for 20 minutes in a microwave oven. Endogenous peroxidase was blocked with 3% H_2O_2. Primary antibodies were incubated overnight. Post-antibody blocking (Immunologic BV, Duiven, Netherlands: post antibody blocking for bright vision plus) was used and the samples were incubated for 40 minutes in the secondary poly-HRP antibody (ImmunoLogic: Poly-HRP-GAM/R/R IgG). Expression levels were detected using a DAB Quanto (Thermo Fisher Scientific, Waltham, MA, USA) system.

Primary antibodies

The primary antibodies were 2SC (1:1000 [crb2005017d] and 1:5000 [crb2005017e], Discovery Antibodies, Billingham, UK), AKR1B10 (1:300, H00057016-M01, Abnova, Taipei, Taiwan), and FH (1:1000, sc-100743, Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Non-commercial 2SC antibody has been previously utilized in analyzing succination levels in series 1.[22] Here, all 197 tumors samples, including the series 1 samples, were analyzed with the commercial 2SC antibody.

Evaluation of staining

Scoring was performed by a pathologist specialized in gynecological pathology (RB). Whole tissue sections of samples with a known FH status were used as positive and negative controls in each staining. If the staining result deviated from those of FH-proficient control samples or was missing in the TMA, the corresponding whole tissue section was stained and analyzed. The three-scale grading system was used to evaluate the cytoplasmic staining in all three antibodies: negative (-), weak and/or discontinuous (+), and strong and continuous (++). For 2SC and AKR1B10, both + and ++ were considered as positive. For FH, only fully negative was considered as negative. Retained staining in endothelial and perivascular cells was used as an internal positive control when scoring FH expression.

Results

Copy number and *FH* mutation analyses

Somatic mono- or biallelic deletion or copy neutral loss of heterozygosity (CN-LOH) in the *FH* locus was detected in 45 unselected uterine leiomyomas (45/1563; 2.9%). See **Supplemental Table 2** for detailed information about copy number analysis results (Supplemental Digital Content 2). One allele was deleted in 41 (41/1563; 2.6%, **Fig. 1A**) and both alleles in four (4/1563; 0.3%, **Fig. 1B**) tumors. Deletion sizes varied from smaller 3.4–60.5Mbp (42/45) to the q-arm (1/45, CN-LOH) and whole chromosome (2/45) deletions. One sample with CN-LOH was identified only through visualization (My1003m1). This sample is hereafter included in the leiomyomas with a monoallelic deletion subgroup.

All 41 samples with monoallelic deletion at the *FH* locus entered direct sequencing of the *FH* coding region. Eight samples harbored a second mutation in the other allele of *FH* (8/41; 19.5%). See **Supplemental Table 2** for additional information about sequencing results (Supplemental Digital

content 2). Five mutations were missense changes predicted to be damaging. One was a splice site mutation previously shown to result in the loss of exon five.[27] Two alterations were synonymous substitutions in the last nucleotides of exons three and four. Both synonymous mutations were shown to result in splicing defects in cDNA sequencing – one led to a shift in the reading frame and the other to the loss of exon four (c.378G>A, p.[Glu126=;Ala128Glyfs*4] and c.555G>A, p.Val127_Gln185del; **Fig. 1C-D**). Taken together, SNP array analysis and Sanger sequencing detected 12 uterine leiomyomas (12/1563; 0.8%) that displayed somatic biallelic *FH* inactivation, four through biallelic deletions and eight through monoallelic deletion and a point mutation in the *FH* coding region. As series 2 also included 27 tumors from known HLRCC patients (20 tumors with biallelic and seven with monoallelic *FH* mutation), it included altogether 72 tumors that had at least one mutation affecting the *FH* locus (72/1590; 4.5%). Thirty-two tumors showed biallelic (32/1590; 2.0%) and 40 monoallelic (40/1590; 2.5%) mutations.

Immunohistochemistry

Altogether 197 tumor samples were included in the immunohistochemical analyses – all 155 samples from series 1 and 42 samples from series 2. Series 1 included 90 tumors from HLRCC patients and 65 sporadic tumors. Series 2 samples were selected based on copy number analysis and *FH* sequencing and included 42 sporadic samples showing mono- (n=15) or biallelic (n=7) *FH* mutation and 20 samples with no *FH* deletion (n=20). Sample selection workflow is presented in **Fig. 2**.

Inactivation of FH was determined indirectly using the 2SC-antibody. In series 1, all FH-deficient tumors from HLRCC-patients showed high succination levels (**Fig. 3, Table 1).** See **Supplemental Table 3** for detailed information about 2SC staining results of series 1 (Supplemental Digital Content 3). All 67 sporadic tumors, including two *MED12* positive tumors derived from two HLRCC patients, displayed no succination (**Fig. 4, Table 1)**. When the staining intensities between the commercial and non-commercial 2SC antibodies were compared, the results were identical for all samples. In series

2, all seven sporadic samples with biallelic *FH* loss showed strong succination (**Fig. 3, Table 2)**. See **Supplemental Table 2** for detailed information about 2SC staining results of series 2 (Supplemental Digital Content 2). Sporadic samples with monoallelic *FH* loss and no deletion in *FH* locus displayed no succination (**Fig. 4, Table 2)**. Overall, the 2SC antibody displayed 100% sensitivity and specificity in both sample series. No sample gave an intermediate staining result.

AKR1B10 showed strong positivity in most HLRCC related FH-deficient uterine leiomyomas (70/88, 80%) (**Fig. 3, Table 1).** See **Supplemental Table 3** for detailed information about AKR1B10 staining results of series 1 (Supplemental Digital Content 3). Weak positivity was seen in 18 samples (18/88, 20%). Two *MED12* positive tumors from two HLRCC patients and all 65 sporadic tumors were negative for AKR1B10 **(Fig. 4, Table 1)**. In series 2, strong AKR1B10 expression was observed in all samples with biallelic loss of *FH* (**Fig. 3, Table 2)**. See **Supplemental Table 2** for detailed information about AKR1B10 staining results of series 2 (Supplemental Digital Content 2). All samples with monoallelic *FH* loss were negative for AKR1B10 (**Fig. 4, Table 2)**. Most samples with no deletion in *FH* were also negative, while one sample displayed strong AKR1B10 expression (**Fig. 5A, Table 2)**. In addition, series 1 and series 2 displayed one and two samples with a few AKR1B10 expressing cells, respectively. These samples were scored as negative. Across both sample series, sensitivity was 100% and specificity 99% for the AKR1B10 antibody.

The loss of FH protein expression was detected in 79/88 (90%) HLRCC-associated tumors (**Fig. 3, Table 1)**. See **Supplemental Table 3** for detailed information about FH staining results of series 1 (Supplemental Digital Content 3). Four samples showed weak and five strong FH expression (9/88, 10%; **Fig. 5B**). Of these nine samples with a false negative result, five had a germline *FH* frameshift mutation c.671_672delAG; p.(Glu224Valfs*25) and four had a germline missense mutation: c.587A>G; p.(His196Arg) in two samples, and c.583A>G; p.(Met195Val) in two samples. In sporadic uterine leiomyomas from series 1, including two *MED12*-positive tumors from two HLRCC

patients, FH displayed strong expression in 65/67 (97%) tumors (**Fig. 4, Table 1)**. Two sporadic tumors showed reduced expression (2/67, 3%). From series 2, all sporadic samples with biallelic *FH* loss displayed loss of FH expression (**Fig. 3, Table 2**). See **Supplemental Table 2** for detailed information about FH staining results of series 2 (Supplemental Digital Content 2). Majority of samples with monoallelic *FH* loss (13/15, 87%) and no deletion in *FH* (15/20, 75%) showed strong FH expression, whereas two and five tumors displayed weak expression, respectively (**Fig. 4, Table 2**). The overall sensitivity was 91% and specificity 100% for the FH antibody.

Discussion

HLRCC patients typically develop several symptomatic uterine leiomyomas, which often require hysterectomy, a treatment eliminating childbearing and having additional adverse outcomes on general health. Other typical HLRCC patient characteristics include early age at onset and strong family history. FH-deficient tumors may display specific morphological features that can in addition to patient characteristics arouse suspicion of HLRCC. These tumor characteristics include large eosinophilic nucleoli surrounded by perinucleolar halos, scattered bizarre nuclei, eosinophilic cytoplasmic inclusions, and staghorn vasculature.[15-17] While these characteristics are suggestive of FH-deficiency, they are not restricted to FH-deficient tumors nor do all FH-deficient tumors display them[16], making them insufficient for clinical diagnosis. Here, we analyzed the feasibility of 2SC, AKR1B10, and FH antibodies in detecting FH-deficiency in uterine leiomyomas. We utilized two unique sample series, the one focusing on HLRCC-associated leiomyomas and the other on unselected tumors.

Fumarate hydratase is one of the enzymes in the tricarboxylic acid cycle, where it catalyzes the hydration of fumarate to malate.[30] Loss of both functional *FH* alleles causes intracellular high-level accumulation of fumarate.[31] Fumarate, in turn, reacts spontaneously with free cysteine sulfhydryl groups on proteins in a Michael addition reaction forming 2SC modifications in a process termed

succination.[32] Thus, biallelic inactivation of *FH* leads to aberrant succination of proteins. We and others have shown that a non-commercial 2SC antibody that detects succinated proteins identifies HLRCC-associated uterine leiomyomas with high sensitivity and specificity.[19,21,22,33] Here, we utilized the same sample cohort (sample series 1) as in our previous study to evaluate the performance of the new commercially available 2SC antibody. Results with the commercial and non-commercial antibodies were 100% concordant. Both antibodies showed strong succination in all HLRCC-associated FH-deficient tumors and no succination in sporadic tumors. Similarly, in unselected leiomyoma series, all tumors with biallelic *FH* loss showed strong succination while tumors with monoallelic *FH* loss or no deletion in *FH* displayed no succination. Overall, 2SC displayed 100% sensitivity and specificity. Scoring was straightforward, with staining being either clearly positive or negative in the tumor cells. Validation in other laboratories is still required, but our results indicate that the 2SC antibody is a robust biomarker for detecting FH-deficient uterine leiomyomas and applicable for diagnostic use.

Aldo-keto reductases are a group of proteins that catalyze oxidation-reduction reactions in the cytosol.[34] Dysfunction of AKR1B10 has been associated with diseases like diabetes mellitus and cancer, including hepatocellular carcinoma.[35] Indeed, AKR1B10 has been considered as a potential biomarker for hepatocellular carcinoma. We have previously shown that uterine leiomyomas with biallelic *FH* loss significantly overexpress *AKR1B10* at the RNA level.[4] Here, we tested whether protein-level expression of AKR1B10 could serve as a putative biomarker for FH-deficient uterine leiomyomas. Immunohistochemistry revealed that AKR1B10 did identify most FH-deficient tumors. However, a clear false positive result was also observed, as one sample with no deletion in *FH* showed strong expression. When compared to 2SC, the staining intensities were slightly variable posing challenges for interpretation and reliable scoring. While the AKR1B10 antibody performed relatively well in the analysis, reduced specificity and uneven staining makes its use in a clinical setting not optimal.

Direct measurement of FH expression to detect potential HLRCC-associated uterine leiomyomas is currently being used in many clinics. This is, however, problematic, as retained expression has been observed in several FH-deficient tumors.[19-21,36] Many *FH* mutations are missense alterations[37,38], which are more likely to result in FH protein expression when compared to other mutation types[18,19,39] as they lead to the production of a stable but inactive enzyme that may still be detectable with immunohistochemistry. Compatible with these findings, we identified nine HLRCC-associated tumors with biallelic *FH* mutation that showed reduced or even strong FH protein expression. In addition, some FH-proficient tumors showed reduced FH expression. Taken together, while FH antibody does identify most FH-deficient tumors, it also produces false negative results as well as equivocal staining pattern indicating that direct FH staining is not optimal for detecting FH-deficient tumors.

Genotyping and sequencing revealed biallelic *FH* inactivation in 12 unselected uterine leiomyomas. All these mutations were somatic. In addition, the series included three previously identified HLRCC patients resulting in the prevalence of 0.6% (3/515). Altogether 27 tumors had been removed from these three HLRCC patients. Majority of these tumors were FH-deficient (20/27; 74%), but several sporadic *MED12* mutated tumors were also detected. This shows that HLRCC-patients develop also sporadic tumors and highlights the need to include more than one tumor in immunohistochemistry when HLRCC is suspected. The total amount of FH-deficient tumors in the unselected series was 2.0% (32/1590), which is in line with previous studies.[16,19,40]

Molecular analyses revealed 33 tumors with monoallelic deletion at the *FH* locus. Of the 15 tumors that were available for immunohistochemistry, none displayed succination. This indicates that one functional *FH* allele is sufficient for normal or close to normal function of the tricarboxylic acid cycle. Similar results have been observed in expression profiling, where myometrium samples from HLRCC

patients showed normal cell metabolism and clustered together with other normal tissue samples.[4,41] This indicates that in addition to *FH*, recurrent 1q deletions observed here and in previous uterine leiomyoma studies[9,42] may target other gene(s) whose loss provides a growth advantage for the cells.

Immunohistochemistry is a cost-effective method to analyze protein expression levels in tumors. A suitable biomarker used for diagnostic purposes should produce reproducible and unambiguous staining results. In hereditary tumor predisposition syndromes, false positive staining leading to unnecessary genetic testing causes anxiety for the individuals whereas false negative results leave patients undetected. Our results show that 2SC, AKR1B10, and FH antibodies all perform relatively well in detecting FH-deficient uterine leiomyomas. Of the three antibodies, 2SC performed best with 100% specificity and sensitivity and straightforward scoring. We propose that when clinical patient characteristics and/or uterine leiomyoma tumor histopathology even in one tumor indicate potential FH-deficiency, 2SC immunohistochemistry should be performed. Occasionally, there may be dozens of leiomyoma-like tumors in a removed uterus and the selection of tumors for microscopic and eventual immunohistochemical analyses relies on macroscopic morphology as well. As syndromic tumors may present conventional morphology and HLRCC patients may develop also sporadic leiomyomas, we suggest analyzing more than one tumor from each patient. Tumor morcellation poses additional challenges for diagnostics and more than one tissue block should be stained. While the likelihood of HLRCC increases when there are several 2SC positive tumors, we suggest that when 2SC positivity is identified even in one tumor or tissue section the patient should be referred to genetic counselling and consideration of germline testing. Targeted *FH* mutation screening on the patient's normal tissue sample (e.g. blood, saliva) should be performed to determine whether one defective allele is in the germline. When a germline mutation – and thus HLRCC syndrome – is diagnosed, family members can be directed to genetic counseling and mutation testing. Identification of HLRCC-patients enables active family planning and regular follow-up for renal cell cancer.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

TA and PV designed the study; TA, JK, and KK performed the molecular analyses and analyzed the data; OU, AA, and OH provided samples; NM, LAA, AK and PV supervised the study and participated in data interpretation; RB evaluated immunohistochemical stainings; TA, JK, and PV wrote the manuscript. All authors read and approved the final paper.

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Figure legends

Fig 1 Copy number analysis and direct sequencing of fresh frozen uterine leiomyomas revealed mutations affecting *FH*. Heatmap displays monoallelic deletion in sample My8025m1 in light blue (**A)** and biallelic deletion in sample My6303m1 in dark blue (**B)** according to the changes detected in Log R ratios and B-allele frequencies. End of chromosome 1 (chr1) q-arm is shown on the left and a figure zoomed into *FH* is shown on the right. *FH* locus is marked with an arrow. **C** A point mutation in the end of exon four (ex4), just before intron four (in4), in sample My6153m1 disrupts the donor splice site and results in skipping of exon four. **D** A point mutation in the last nucleotide of exon three (ex3) in sample My1003m1 leads to a loss of a canonical splice site and activation of a cryptic donor splice site in the beginning of intron three (in3). It leads to an insertion (ins) of seven nucleotides from the intron to the messenger RNA (mRNA), and thus to a shift in the reading frame and a premature termination codon in exon four. Genomic DNA sequence shows the mutation in the nuclear DNA. Complementary DNA (cDNA) sequence and schematic depiction show how the splice site mutation affects mRNA. Mutation sites are marked with red dots and a stop codon with a red asterix. Figures A and B obtained from Partek software.

Fig 2 Flowchart of the sample selection process for uterine leiomyomas entering immunohistochemical analyses with 2SC, AKR1B10, and FH antibodies. In total, 197 formalin-fixed paraffin-embedded (FFPE) tumor samples (90 tumors from HLRCC patients and 107 sporadic tumors including seven with somatic biallelic and 15 with somatic monoallelic loss of *FH*) were analyzed. FFPE samples highlighted with orange, fresh frozen (FF) samples with blue. *Two corresponding FFPE samples from one HLRCC patient in series 2 were analyzed among HLRCC samples in series 1.

Fig 3 Representative staining results with 2SC, AKR1B10, and FH antibodies in FH-deficient uterine leiomyomas. Leiomyoma from an HLRCC patient with one germline and one somatic *FH* mutation, as well as a tumor with somatic biallelic *FH* loss, show positivity for 2SC and AKR1B10 but no expression for FH. All images with ×400 magnification.

Fig 4 Representative staining results with 2SC, AKR1B10, and FH antibodies in FH-proficient uterine leiomyomas. Strong FH expression but no succination or AKR1B10 expression were detected in a sporadic FH-proficient sample, tumor from an HLRCC patient (with a germline *FH* mutation and a somatic *MED12* mutation), tumor with monoallelic *FH* loss, and tumor with no deletion in the FH locus. All images with ×400 magnification.

Fig 5 False positive and false negative staining results with AKR1B10 and FH antibodies. **A** False positive staining with the AKR1B10 antibody in a sample with no *FH* deletion. **B** False negative staining of FH-deficient HLRCC-associated leiomyoma with the FH antibody. All images with ×400 magnification.

Supplemental Digital Content

Ahvenainen_SDC_1.xls

Supplemental Digital Content 1

Supplemental Table 1. Uterine leiomyomas removed from HLRCC patients in series 2.

Ahvenainen_SDC_2.xls

Supplemental Digital Content 2

Supplemental Table 2. Copy number analysis at the FH locus, FH mutation screening, and 2SC, AKR1B10, and FH immunohistochemical staining results for sporadic uterine leiomyomas in series 2.

Ahvenainen_SDC_3.xls

Supplemental Digital Content 3

Supplemental Table 3. Immunohistochemistry results for HLRCC-associated and sporadic uterine leiomyomas of series 1.