non-small cell lung carcinoma (NSCLC) may be challenging.^{8,9} The latter shows striking pleomorphism in contrast to the monotonous appearance of *SMARCA4*-DTS, is frequently positive for Hepar-1 and cytokeratin-7, and usually shows some degree of glandular differentiation which is always absent in *SMARCA4*-DTS.⁸ Nevertheless, *SMARCA4*-DTS and *SMARCA4*-deficient NSCLC show considerable overlap in their clinicopathological profiles: male preponderance, association with smoking, pattern of metastases, poor clinical outcomes, histomorphology, immunoprofile, and molecular alterations such as concurrent *TP53* and *KRAS* alterations.^{5,8} Whether these two tumours represent spectral ends of the same entity needs further clarification.

We document, for the first time, an innocuous presentation of *SMARCA4*-DTS wherein a small tumour (~4 cm grossly) was incidentally detected in the soft tissue of chest wall around an ICD insertion site in a patient suffering from chronic empyema thoracis of uncertain aetiology for over 10 months. We believe it is unlikely that the *SMARCA4*-DTS was masquerading as chronic empyema as the initial computed tomography images do not show any mass lesion. The lung parenchyma was completely clear except for smoking related parenchymal changes. Although an autopsy was refused in this patient, the rapidly fatal outcome after development of tumour highlights its aggressiveness. A link with chronic infection/inflammation as an inciting/contributing factor for *SMARCA4*-DTS has not been noted previously and should be explored.

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Concordance of p16, FH, and alpha-SMA expression with the fumarate hydratase gene mutational status in sporadic and hereditary piloleiomyomas

Sir,

Piloleiomyoma (PLM) is a rare cutaneous leiomyoma (CL) arising from arrector pili muscles. It appears as a firm, skin-coloured to light brown papule or nodule, distributed over the trunk and extremities, occasionally on the face and neck.¹ It occurs more often as an isolated cutaneous leiomyoma (ICL), representing a sporadic, non-familial condition. Less frequently, PLM occurs as multiple cutaneous leiomyomatosis (MCL), both in hereditary (H-MCL) and non-hereditary settings (NH-MCL). H-MCL represents the cutaneous hallmark of hereditary leiomyomatosis and renal cell cancer (HLRCC), an autosomal dominant syndrome that affects heterozygous carriers of germinal mutation (MUT) in the *FH* gene. HLRCC clinically predisposes to MCL (both diffuse and segmental pattern), multiple uterine leiomyomas, renal cell cancer and pheochromocytomas. Uterine and cutaneous leiomyosarcoma, ovarian mucinous cystadenoma, bladder, breast, adrenal and testicular cancers have been also described in FH MUT individuals; it remains to be established whether these tumours are effectively associated with HLRCC.^{1,2} The genetic basis of H-MCL is well known; conversely, the pathogenic mechanism of non-hereditary PLM occurring in wild-type FH gene carriers is still unclear.^{1–6} As a rule, wild-type FH subjects manifest a solitary CL (ICL). NH-MCL have been reported only in four wild-type FH subjects, as a segmental pattern, without unequivocal genetic and immunohistochemical studies (Supplementary Table 1, Appendix A).^{3–6} The diffuse pattern of NH-MCL has never been described.

Recent findings suggest that FH immunoreactivity is conserved in most sporadic PLMs; conversely, CLs of HLRCC patients show negative or weak positive immunostaining for FH.⁶ It is well known that benign cutaneous smooth muscle tumours, including PLMs, show immunoreactivity for Alpha-smooth muscle actin (Alpha-SMA). Nevertheless, the expression pattern of alpha-SMA in hereditary and sporadic PLMs is unknown.^{1,2} p16 is one of the most common human tumour suppressor genes. There are some data on the expression of p16 in extra-cutaneous leiomyomas, in melanoma and non-melanoma skin cancers; nonetheless, its expression in PLM is currently completely unexplored.^{7–9}

We report the clinical and genetic findings of eight cases of sporadic and familial CL in which FH, p16 and alpha-SMA

Table 1 Clinical, genetics, IHC (FH, p16 and alpha-SMA) findings of our non-hereditary and hereditary PLM patients

Patient no.	Clinical findings	Family history	FH gene ^a	IHC FH expression ^b	IHC p16 expression ^b	IHC alpha-SMA expression ^b
1	46-year-old Caucasian woman (Italian) with 1 year history of multiple PLMs on her upper and lower limbs, bilaterally	Positive for UMs Negative for PLMs and RCC	WT	Positive (low)	Positive	Positive (low)
2	46-year-old Caucasian man (Italian) with isolated PLM of the back of recent onset	Negative for PLMs, RCC, UMs	WT	Positive	Positive	Positive (low)
3	48-year-old African woman with isolated PLM of the chest of recent onset	Negative for PLMs, RCC, UMs	WT	Positive (low)	Positive (low)	Negative
4	60-year-old Caucasian woman (Ukrainian) with isolated PLM of the chest of recent onset; multiple UMs since 30 years of age	Positive for UMs Negative for PLMs and RCC	WT	Positive (low)	Positive (low)	Negative
5	36-year-old Caucasian woman (Italian) with a history of over 15 years of multiple PLMs on her lower limbs, bilaterally; multiple UMs since 22 years of age (multiple myomectomy interventions)	Positive for PLMs and UMs (mother) and colon carcinoma (maternal grandfather) Negative for RCC	Heterozygous mutation c.268delA (p.Thr90Profs*10) on exon 3 ^c	Negative	Negative	Positive
6	61-year-old Caucasian woman (Italian) with multiple PLMs since 30 years of age on her trunk, upper and lower limbs, bilaterally; multiple UMs since 30 years of age (hysterectomy at 46 years of age); breast cancer (ductal carcinoma of the left breast) diagnosed at 66 years of age	Positive for PLMs (father and paternal aunt) Negative for RCC and UMs	Heterozygous mutation c.944T>C (p.Leu315Pro) on exon 7	Negative	Negative	Positive
7	37-year-old Caucasian women (Italian) with multiple PLMs since adolescence on her trunk, upper and lower limbs, bilaterally; multiple UMs since 20 years of age (hysterectomy at 32 years of age); uterine leiomyosarcoma and RCC at 32 years of age	Positive for PLMs (father) and UMs (paternal aunts) Negative for RCC	Heterozygous mutation c.1391-2delA on intron 9 ^c	Negative	Negative	Positive
8	32-year-old Caucasian woman (Italian) with multiple PLMs since adolescence on her upper and lower limbs, bilaterally; multiple UMs since 20 years of age (myomectomy at 25 years of age)	Positive for PLMs (father) and UMs (paternal aunt, cousin, grandmother) Negative for RCC	Heterozygous mutation c.875_876insAA (p.Val293Argfs*37)	Negative	Negative	Positive

^a Methods of genetic analysis: Genomic DNA was extracted from peripheral whole blood (Qiagen EZ1 DNA Blood 350 µL Kit) according to manufacturer's instructions. PCR amplification of exons from 1 to 10 of *FH* gene was performed with AmpliTaq Gold @360 DNA Polymerase (Applied Biosystems, USA) using a Touch-down PCR protocol in a final volume of 25 µL. Sanger sequencing was done using BigDye Terminator v1.1 Cycle Sequencing Kit on a 3130xl Genetic analyzer (Applied Biosystems). MLPA assay of *FH* gene (100ng germline DNA) was performed for all cases using SALSA MLPA kit P198-A2 (MRC-Holland, The Netherlands) and analysed using [Coffalyser.NET](http://www.coffalyser.net) software (MRC-Holland). The mutation nomenclature is according to HGVS Nomenclature for the Description of Sequence Variants (<http://www.hgvs.org/mutnomen>). Genbank mRNA ref. seq: NM_000077.4; NM_000143.3.

^b FH and alpha-SMA expression were investigated deparaffinising and hydrating sections with xylene and graded ethanol, respectively. Heat-induced antigen retrieval was performed, and sections were incubated 1 h with rabbit polyclonal antibody anti-FH (ab95947, 1:200; Abcam, USA) and monoclonal mouse anti-alpha SMA (1:800; Sigma, USA). Then slides were incubated with biotinylated anti-rabbit and anti-mouse antibody (Vector Laboratories, USA) and immunoreactivity was detected using Vector DAB Substrate Kit (Vector Laboratories). Counter stain was performed using Mayer's haematoxylin (Sigma). Analysis of p16 localisation was performed with an automated stainer (Benchmark Ultra; Ventana Medical Systems, USA) using anti-p16 mouse monoclonal primary antibody (1.0 µg/mL; Ventana Medical Systems). Images were captured by brightfield microscopy at 100× microscopic magnification and three images from each slide were obtained for quantification. FH, p16 and alpha-SMA staining was analysed using IHC profiler plugin of the ImageJ 1.52a software, while quantification was performed using IHC ToolBox plugin for ImageJ software.^{11,12}

^c Pathogenic FH mutations never described previously in the literature. Several *in silico* splicing prediction models support their pathogenicity. In the first case (Patient 5) the results of the splicing analysis (Splice Predictor, Splice View, NetGene, GeneScan) suggest the loss of the splicing acceptor site. In the second case (Patient 7), the variant causes a frameshift with consequent formation of a truncated protein. The splicing prediction *in silico* (Human Splicing Finder, Mutation Taster, BDGP, NetGene2, Cryp-Skip) suggests the possible reduction in the effectiveness of the acceptor site of exon 3 and therefore an effect of the mutation on gene processing cannot be excluded.

IHC, immunohistochemistry; PLM, piloleiomyoma; RCC, renal cell cancer; UM, uterine myoma; WT, wild-type.

staining has been investigated, to examine the relationship between its expression and *FH* gene status.

After the collection of personal and family history and the acquisition of informed consent, all subjects were investigated for the presence of a *FH* germline mutation. Conventional haematoxylin/eosin staining was performed on tissue samples of all patients. *FH* and p16 protein expression were examined by immunohistochemistry (IHC); alpha-SMA expression was evaluated with both immunofluorescence (IF) and IHC in order to quantify and accurately localise protein expression. IHC and IF quantification was performed using the ImageJ 1.52a software (<https://imagej.nih.gov/ij/>); results were expressed as the percentage of positive stain/area in three different pictures of the same samples $\pm$ SD. Statistical analysis was performed with a two-tailed paired Student's t-test using GraphPad Prism 6 software (GraphPad Software, USA). $p < 0.05$ was taken as statistically significant.

Clinical and genetic characteristics of patients are summarised in Table 1, together with results for IHC *FH*, p16, and alpha-SMA expression. We identified germline heterozygous mutations of *FH* gene in Patients 5, 6, 7 and 8 (MUT patients, Table 1; Supplementary Fig. 1, Appendix A). Pathogenic mutations in Patients 5 and 7 have not been previously described. Patients 1, 2, 3 and 4 were carriers of a wild-type *FH* gene.

All patients showed at least one histologically confirmed PLM. Microscopic examination demonstrated non-encapsulated tumours of the superficial dermis, composed of bundles of closely packed cells with uniform elongated nuclei and eosinophilic cytoplasm. Necrosis and mitotic figures were absent. Patients 1, 5, 6, 7 and 8 had MCL with a diffuse pattern, mainly on extremities. Patients 2, 3 and 4 manifested only a single ICL of the trunk.

FH and p16 IHC staining showed positive protein expression with a similar pattern in all sporadic PLMs; on the contrary, in all samples from *FH* MUT patients we observed a statistically significant reduction in *FH* and p16 IHC expression (Table 1, Fig. 1). Moreover, in wild-type *FH* patients we observed statistically significant reduced alpha-SMA expression in comparison with *FH* MUT patients. Conversely, PLM cells of MUT subjects showed high-intensity staining for alpha-SMA (Table 1, Fig. 2A,B,F–H).

No differences between the various samples were observed regarding p16, *FH* and alpha-SMA staining in the normal skin tissue surrounding PLMs. In all skin samples, we confirmed *FH*, p16 and alpha-SMA expression in skin vessels, representing the internal positive controls (Fig. 1I,L; Fig. 2I). In order to precisely localise the alpha-SMA expression, we performed IF on both sporadic and hereditary PLMs (Fig. 2C,D), showing a cytoplasmic stain in

Fig. 1 Histological and IHC images of PLM from Patient 1 (A,C,F; sporadic CL) and Patient 5 (B,D,G,I,L; hereditary CL). H&E staining (A,B) and corresponding p16 IHC (C,D) and FH IHC (F,G). p16 (E) and FH (H) IHC quantification (* $p < 0.05$). Data are mean $\pm$ SD of three different images of the same sample. Black arrows indicate positive internal controls both for p16 IHC (I) and FH IHC (L).

Fig. 2 Histological, IF and IHC images of PLM from Patient 1 (A,C,F,I; sporadic CL) and Patient 5 (B,D,G; hereditary CL). H&E staining (A,B), corresponding IF (C,D) and IHC (F,G) of alpha-SMA expression. IF (E) and IHC (H) quantification of alpha-SMA expression in sporadic and hereditary PLMs respectively ($***p < 0.001$). Data are mean $\pm$ SD of three different pictures of the same sample. Black arrows indicate positive internal controls for alpha-SMA IHC expression (I). For IHC methods see Table 1. IF methods: Four- μ m sections of formalin-fixed, paraffin-embedded tissue were deparaffinised with xylene and graded ethanol, respectively. Heat antigen retrieval was performed; slides were incubated for 2 hours with monoclonal mouse anti-antibody alpha-SMA (1:800; Sigma, USA) followed by incubation with a secondary antibody conjugated to the Alexa Fluor 546 fluorophore (1:600; Life Technologies, USA). Images were captured using a fluorescence microscope (DM5500B; Leica, Germany); quantification of antigen expression was performed using ImageJ Software.

MUT tumour cells, strongly reduced in sporadic PLMs (Fig. 2E).

Germline mutations of *FH* gene are actually the most important genetic susceptibility factors associated with H-MCL, even if the exact pathogenic mechanism of sporadic CL (both NH-MCL and ICL) is still unknown.^{1–6}

It has been hypothesised that in HLRCC patients the loss of FH can lead to fumarate accumulation, facilitating a pseudo hypoxic state, with aberrant activation of hypoxia response pathways. Fumarate is able to inhibit proline hydroxylation of the hypoxia-inducible factor HIF; the unhydroxylated form of HIF is not recognised by the E3 ubiquitin ligase VHL (von Hippel–Lindau tumour suppressor protein, which directs the proteasomal degradation of HIF) and is thus stabilised. HIF can activate the transcription of several genes, including VEGF and other protumorigenic growth factors (i.e., GLUT1, PDGF, TGF- α and TGF- β). The latter regulates the alpha-SMA expression.¹

Llamas-Velasco *et al.* studied the role of IHC in FH for the diagnostic assessment of cutaneous sporadic and hereditary PLMs. About 83% of cutaneous PLMs from HLRCC patients presented negative FH staining; the remaining 17% were

weakly positive for FH. About 75% of sporadic PLMs showed positive FH staining. The arrector pili muscles from HLRCC patients maintained FH positive staining, suggesting the central role of heterozygosity loss of the *FH* gene in the tumorigenesis of PLMs.⁶

p16 is a protein encoded by *CDKN2A* gene that plays an important role as a tumour suppressor gene both in germline and somatic settings. Due to its key role in cell cycle control, other than regulation of senescence, apoptosis, cell invasion and angiogenesis, p16 represents one of the most frequently mutated genes detected in a wide range of human cancers. Although p16 expression has been widely investigated in many tumours, including uterine smooth muscle tumours (SMTs), there are no data in the literature concerning sporadic and hereditary PLMs. As regards extra-cutaneous SMTs, IHC p16 overexpression is common in malignant and atypical uterine SMTs, but its diagnostic and prognostic value in these SMTs is not yet well established.^{7–9} In non-melanoma skin tumours, IHC studies have demonstrated that both precancerous lesions, squamous and basal cell carcinomas usually show a high proliferation rate in combination with p16 overexpression and lack of retinoblastoma

protein (pRB)-phosphorylation. This indirectly could represent an aberrant function of pRB pathway, suggesting a different pathogenic mechanism involved in FH mutated cancers.^{7,9} Regarding alpha-SMA, recent data suggest that overexpression of Retinoblastoma-binding protein 2 (RBP2) can increase alpha-SMA levels; conversely, the gene knockdown of RBP2 expression decreases levels of alpha-SMA.¹⁰ RBP2 is a nuclear histone demethylase implicated in epigenetic transcription regulation of a wide range of genes through the pRB and p16 tumour suppressor proteins. An aberrant function of pRB pathway could lead to down-regulation of RBP2; this, in turn, would decrease the HIF expression levels, with a consequent reduction in alpha-SMA expression.¹⁰

We report for the first time a detailed p16, FH, and alpha-SMA protein expression study of cutaneous leiomyoma in FH genotyped patients. We observed that p16, FH, and alpha-SMA expression patterns appear to be correlated with the genotypic status of the *FH* gene. Our IHC FH findings are in agreement with what has already been described in the literature in patients affected by sporadic PLM (positive expression) and HLRCC-PLM (negative or weak positive).⁶ Regarding alpha-SMA expression, we observed a negative IHC and IF expression in sporadic PLM, unlike HLRCC-PLMs that showed an intense reactivity for this protein.

Our data could suggest a possible new aetiopathogenetic hypothesis for NH-CL. These findings would support a different tumourigenic mechanism of CL in wild-type FH patients, compared to FH germline mutation carriers. Since PLM of wild-type FH subjects showed FH and p16 overexpression and concomitant negative expression of alpha-SMA, the potential pathogenic role of an aberrant (inactive, non-functional) Rb-pathway could be hypothesised, similar to that reported in the literature for other skin and non-skin tumours.⁷⁻⁹

Here we also describe for the first time clinical, IHC, IF and genetic features of a diffuse MCL in a wild-type FH patient (NH-MCL, Patient 1).

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.pathol.2019.05.006>.

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Molecular analysis of melanocytic naevus arising from ovarian mature teratoma

Sir,

Melanocytes are cutaneous or extracutaneous, such as uveal melanocytes, and they are all considered of neural crest origin except for the retinal pigmented epithelial cells, which originate from neural ectoderm of the developing forebrain.¹ Melanocytic naevi are clonal proliferations of melanocytes comprising different subtypes such as congenital, acquired, blue and Spitz naevi, each with a different genetic background and driver mutation: acquired naevi harbour *BRAF* mutations; congenital naevi *NRAS* and, to a lesser extent, *BRAF* mutations; blue naevi *GNAQ* mutations; and Spitz naevi *HRAS* mutations.² In melanomas, *BRAF* mutations are more common in tumours not arising in the context of chronic sun damage.³ Melanocytic tumours usually involve the skin, while extracutaneous sites are rarer. An extremely rare phenomenon is the development of melanocytic tumours inside