Smooth muscle differentiation identifies two classes of poorly differentiated pleomorphic sarcomas with distinct outcome

Gaëlle Pérot[1,2], Jean Mendiboure[3], Véronique Brouste[3], Valérie Velasco[1], Philippe Terrier[4], Sylvie Bonvalot[5], Louis Guillou[6], Dominique Ranchère-Vince[7], Alain Aurias[1,2], Jean-Michel Coindre[1,2] and Frédéric Chibon[1,2]

[1]Institut Bergonié, Department of Pathology, Bordeaux, France; [2]INSERM U916, Bordeaux, France; [3]Institut Bergonié, Clinical and Epidemiological Research Unit, Bordeaux, France; [4]Institut Gustave Roussy, Department of Pathology, Villejuif, France; [5]Institut Gustave Roussy, Department of Surgery, Villejuif, France; [6]University Institute of Pathology, CHUV, Lausanne, Switzerland and [7]Léon Bérard Center, Department of Pathology, Lyon, France

The clinical relevance of accurately diagnosing pleomorphic sarcomas has been shown, especially in cases of undifferentiated pleomorphic sarcomas with myogenic differentiation, which appear significantly more aggressive. To establish a new smooth muscle differentiation classification and to test its prognostic value, 412 sarcomas with complex genetics were examined by immunohistochemistry using four smooth muscle markers (calponin, h-caldesmon, transgelin and smooth muscle actin). Two tumor categories were first defined: tumors with positivity for all four markers and tumors with no or incomplete phenotypes. Multivariate analysis demonstrated that this classification method exhibited the strongest prognostic value compared with other prognostic factors, including histological classification. Secondly, incomplete or absent smooth muscle phenotype tumor group was then divided into subgroups by summing for each tumor the labeling intensities of all four markers for each tumors. A subgroup of tumors with an incomplete but strong smooth muscle differentiation phenotype presenting an intermediate metastatic risk was thus identified. Collectively, our results show that the smooth muscle differentiation classification method may be a useful diagnostic tool as well as a relevant prognostic tool for undifferentiated pleomorphic sarcomas.

Malignant soft-tissue sarcomas are rare tumors, accounting for 1 to 2% of all adult cancers. This rare and vastly heterogeneous group of malignant soft-tissue sarcomas is mainly classified according to clinical and histological features and to an eventual line of differentiation.[1] Molecular approaches have described three main genetics in these soft-tissue sarcomas: first, reciprocal translocations like in synovial sarcomas, second, specific mutations as observed in rhabdoid tumors, and finally, complex genomic profiles.[1–3] This last category is composed of tumors with high-level amplifications of chromosome 12 encompassing *MDM2* and *CDK4* loci (eg, well-differentiated and undifferentiated liposarcomas) (20% of cases)[3–5] and of tumors with very complex genomic imbalances (80% of cases), including mainly leiomyosarcomas and undifferentiated pleomorphic sarcomas.[4,5]

Leiomyosarcomas correspond to 10–15% of soft-tissue sarcomas and display a strong smooth muscle differentiation.[6] They are frequently located in the retroperitoneum, and less frequently in the limbs.[6] Leiomyosarcomas are tumors of poor prognosis with a metastatic rate of 40–45% and a 5-year survival rate of 10–64% depending on their location.[6] Undifferentiated pleomorphic sarcomas are tumors with no specific line of differentiation.[7] They are predominantly observed in the limbs and present a slightly better prognosis with a 5-year survival rate

ranging between 50% and 70%, and local recurrence and metastasis rates of 35–50% and 30–50%, respectively.[7] Undifferentiated pleomorphic sarcomas account for 15% of soft-tissue sarcomas and are currently diagnosed by exclusion.[7] Many studies have re-evaluated and questioned the existence of this type of sarcoma according to the hypothesis that undifferentiated pleomorphic sarcomas could actually represent a common morphologic phenotype shared by various poorly differentiated soft-tissue sarcomas.[8–10] The clinical relevance of a better method of diagnosing undifferentiated pleomorphic sarcomas has been demonstrated, especially in cases of undifferentiated pleomorphic sarcomas with myogenic differentiation, which seem to be significantly more aggressive.[8,9]

In a previous study, we demonstrated that the *MYOCD* gene is amplified and overexpressed in a large proportion of leiomyosarcomas. We have also shown that *MYOCD* expression level controlled smooth muscle differentiation protein expression and had an impact on cell migration in sarcomas.[11] In the present study, 412 sarcomas with complex genetics were reclassified using an immunohistochemical score of smooth muscle differentiation and the impact of this classification on metastatic outcome has been tested. The smooth muscle differentiation level was evaluated in all tumors by immunohistochemistry, using four smooth muscle markers (calponin, transgelin, h-caldesmon (h-CD) and smooth muscle actin (SMA)). The prognostic value of this classification was compared with the histological one.

Materials and methods

Tumor Samples and Histological Classification

Four hundred and twelve soft-tissue sarcomas were selected according to their histological subtypes. Cases were issued from the archives of the Department of Pathology of the Institut Bergonié and from the shared database of the French Sarcoma Group (https://conticabase.sarcomabcb.org). All cases have been systematically reviewed by the pathologists of the French Sarcoma Group according to the World Health Organization recommendations for bone and soft-tissue tumors.[1,6,7] As such, tumors were initially classified into four categories: undifferentiated pleomorphic sarcomas ($n = 148$), leiomyosarcomas ($n = 190$), pleomorphic liposarcomas ($n = 13$) and myxofibrosarcomas ($n = 61$). Pleomorphic liposarcomas and myxofibrosarcomas could represent a differential diagnosis of undifferentiated pleomorphic sarcomas. Cases presented in this study did not present significant differences in terms of metastatic risk compared with other undifferentiated sarcomas (Fine and Gray's model, data not shown). These tumors were thus included in the undifferentiated pleomorphic sarcomas group for the purpose of the

current analyses, leading to two final histological groups: the undifferentiated pleomorphic sarcomas group ($n = 222$) and the leiomyosarcomas group ($n = 190$). Diagnosis, histological, patient and clinical data are presented in Supplementary Table S1. Clinicopathological data summary is presented in Supplementary Table S2. Median follow-up was 10.68 years (95% CI: 9.98; 12.15). Samples used in this study are part of the Biological Resources Center of Institut Bergonié (CRB-IB). In accordance with the French Public Health Code (articles L. 1243-4 and R. 1243-61), the CRB-IB has received the agreement from the French authorities to deliver samples for scientific research (number AC-2008-812, on February 2011). These samples were obtained from regular patient care and were requalified for research. The project was approved by the Committee of Protection of Individuals (CPP Aquitaine).

Immunohistochemistry

The four hundred and twelve tumors were analyzed on tissue microarrays. Each case was represented by three spots of $4 \mu m$ thick and $1 mm$ in diameter. Tissues were deparaffinized in xylene and rehydrated in a series of ethanol baths. For antigen retrieval, two different protocols were used: for anti-CNN1 and anti-CALD antibodies, slides were incubated in DAKO Real Target Retrieval Solution (pH 6) (S2031; DAKO, Carpinteria, CA, USA), for 20 min in a microwave oven, and for the anti-SMA antibody, we used a solution of $CaCl_2$ 0.3% in PBS1 × with 0.1% of trypsin for 15 min. Lastly, for the anti-TAGLN antibody no antigen retrieval was performed. The primary antibodies and dilutions used in this study are as follows: anti-calponin (CALP, 1:100, M3556; DAKO), anti-transgelin (SM22a, 1:500, ab14106; Abcam, UK), anti-h-CD (1:50, M3557; DAKO) and anti-SMA (Clone1A4, prediluted; A2547; Sigma-Aldrich, St Louis, MO, USA). All primary antibodies were incubated for 30 min at room temperature. Finally, revelation was achieved using the DAKO EnVision™ Detection Kit, peroxidase/DAB and rabbit/mouse (K5007; DAKO). Immunohistochemical pictures were taken using a Leitz DMRB microscope (Leica, Nanterre, France) and a DS-Ri1 camera (Nikon, Melville, NY, USA).

Smooth Muscle Differentiation Classification

The smooth muscle differentiation method of classification is based on immunohistochemistry results obtained for the four smooth muscle markers (h-CALD, CNN1, SMA, TAGLN) as previously used:[11] for each marker a strong and global positivity was considered as a score of 2, a low or a partial positivity as a score of 1 and a negative labeling as a score of 0 (Figure 1). A tumor was classified as a leiomyosarcoma only if it displayed a complete differentiation phenotype, that is, if it presented a
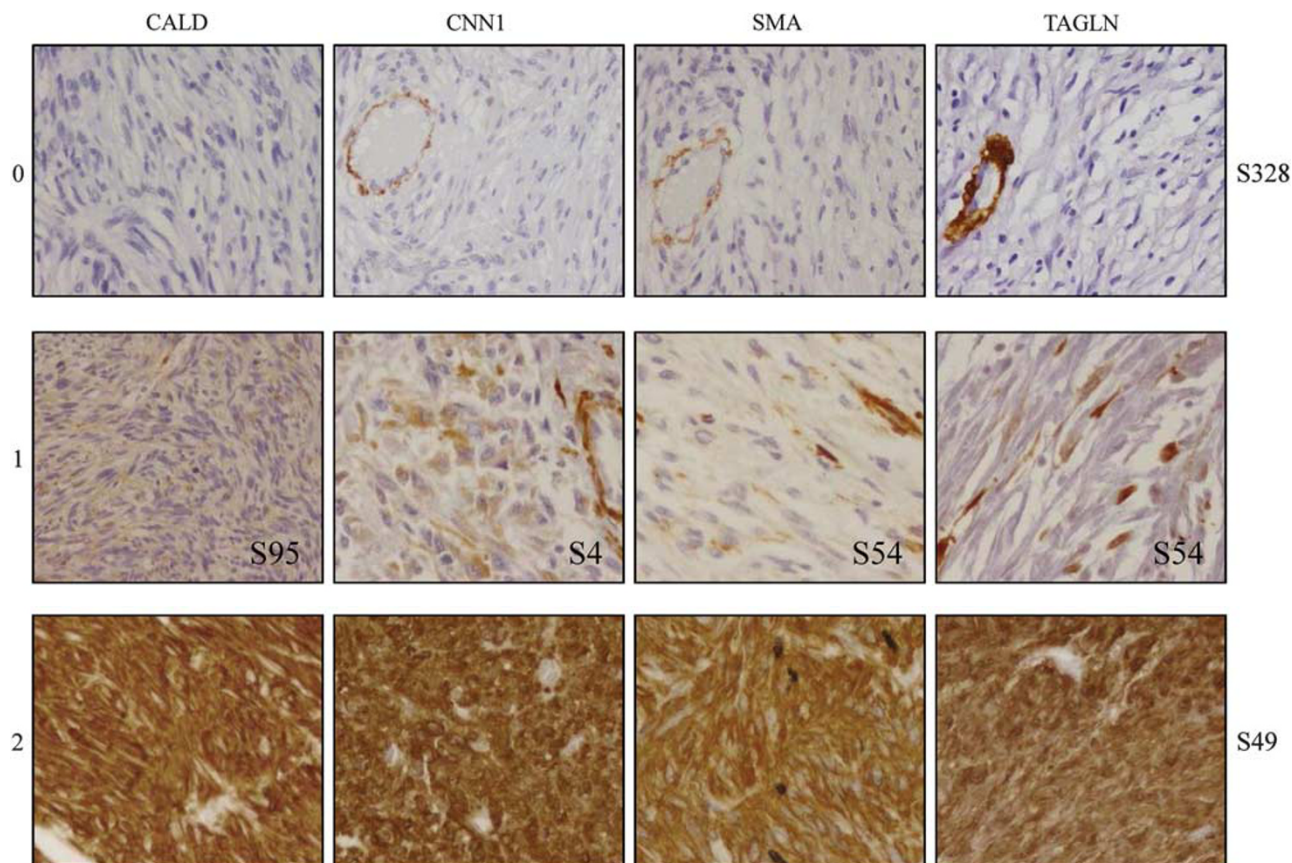
Figure 1 Smooth muscle-related gene expression studied by immunohistochemistry. Examples of immunohistochemistry results obtained for four smooth muscle-related proteins (SMA, CALD, TAGLN and CNN1) in four tumors. The labeling scores (0, 1 or 2) are indicated on the left of the figure. Magnification: × 400.

score of at least 1 for each of the four antibodies used; otherwise, it was considered as an undifferentiated pleomorphic sarcoma. A smooth muscle differentiation score was then calculated by adding, for a given tumor, the immunolabeling intensities for the four proteins (for each marker: score was equal to 0, 1 or 2) to obtain the final score, ranging from 0 to 8. This score was used to establish different subgroups of undifferentiated pleomorphic sarcomas.

Statistical Methods

The cumulative incidence of metastasis was analyzed according to Fine and Gray's model.[12] The event of interest was the metastasis, and competing risks taken into account were local recurrence and death. Multivariate analysis was carried out with Fine and Gray's model using a backwards procedure. Variables associated with survival with a P-value inferior to 0.20 in the univariate analysis were included in the multivariate regression.

Comparison of demographic, clinical and pathological data between the different tumor classification methods were made using χ^2 or Fisher's exact test for categorical variables and Student's t-tests for continuous variables. $P < 0.05$ indicated statistical significance.

Array-CGH

Seventy-eight cases were studied by array-CGH in a previous study.[13] As described previously, genomic profiles were divided into groups: a first group with few alterations mainly involving the full chromosome arm or entire chromosomal gain or loss and a second group with a high level of chromosomal complexity. The first group was called the 'complex/arm-type' genomic profile, and the second group, the 'complex/rearranged-type' genomic profile.

Results

Smooth Muscle Differentiation and Tumor Reclassification

The smooth muscle differentiation level of 412 soft-tissue sarcomas with complex genetics was evaluated by immunohistochemistry using four markers: calponin, transgelin, h-CD and SMA (Figure 1): 46% of tumors were negative for all markers, 13%

expressed one marker, 9% expressed two markers, 10% expressed three markers and 22% were positive for all markers (Table 1). SMA was positive in 40% of tumors, h-CD in 25%, calponin in 39% and transgelin in 48% (Table 1). Intensities of labeling are presented in Supplementary Table S3.

According to the immunohistochemical results, a tumor was classified as a leiomyosarcoma (hereafter referred as scoring leiomyosarcoma) only if it displayed a complete smooth muscle phenotype, that is, if it presented a positive labeling for each of the four antibodies. Other tumors presenting at least one negative marker were considered as undifferentiated pleomorphic sarcoma (hereafter referred as

Table 1 Immunohistochemical and classification data

	N	%
Smooth muscle actin		
Negative	249	60
Positive	163	40
Caldesmon		
Negative	308	75
Positive	104	25
Calponin		
Negative	251	61
Positive	161	39
Transgelin		
Negative	215	52
Positive	197	48
No. of positive markers		
0	187	46
1	52	13
2	38	9
3	43	10
4	92	22
Smooth muscle differentiation classification		
sLMS	92	22
sUPS	320	78
Histological classification		
hLMS	190	46
hUPS	222	54
Histological/smooth muscle differentiation classifications		
hsLMS	89	22
hLMS→sUPS	101	24
hUPS→sLMS	3	1
hsUPS	219	53

Abbreviations: sLMS, tumors classified as leiomyosarcomas by the smooth muscle differentiation classification; sUPS, tumors classified as undifferentiated pleomorphic sarcomas by the smooth muscle differentiation classification; hLMS, tumors histologically classified as leiomyosarcomas; hUPS, tumors histologically classified as undifferentiated pleomorphic sarcomas; hsLMS, tumors classified as leiomyosarcomas by both classification methods; hsUPS, tumors classified as undifferentiated pleomorphic sarcomas by both classification methods; hLMS→sUPS, tumors histologically classified as leiomyosarcomas but reclassified as undifferentiated pleomorphic sarcomas by the smooth muscle differentiation classification; hUPS→sLMS, tumors histologically classified as undifferentiated pleomorphic sarcomas but reclassified as leiomyosarcomas by the smooth muscle differentiation classification.

scoring undifferentiated pleomorphic sarcomas). According to the smooth muscle differentiation classification method, the series was composed of 92 scoring leiomyosarcomas and 320 scoring undifferentiated pleomorphic sarcomas (Table 1). Histological and smooth muscle differentiation classification methods gave the same classification to 308 tumors (75%), whereas 104 tumors (25%) were reclassified with the smooth muscle differentiation classification (Table 1). Among the 104 reclassified tumors, the vast majority (101/104: 97%) was originally histologically classified as leiomyosarcoma (hereafter referred as histological leiomyosarcoma) but reclassified as scoring undifferentiated pleomorphic sarcomas by the smooth muscle differentiation method.

What is the Prognostic Impact of the Smooth Muscle Differentiation Reclassification?

We tested whether this smooth muscle differentiation classification had a prognostic impact on metastatic outcome. Univariate analysis showed that this classification method has a significant prognostic value (subdistribution hazard ratio = 2.62; 95% CI: 1.89; 3.62; $P < 0.0001$) (Tables 2 and 3 and Figure 2). Moreover, in the multivariate analysis, the smooth muscle differentiation classification exhibited the strongest prognostic impact and thus outperformed all other prognostic factors, including histological diagnosis (subdistribution hazard ratio = 2.077; 95% CI: 1.34; 3.22; $P = 0.0011$) (Table 4).

To go further in the comparison of the prognostic values between the two classification methods, metastatic outcomes between the leiomyosarcomas reclassified as undifferentiated pleomorphic sarcomas by the smooth muscle differentiation method and the tumors equally classified by both methods were compared (Figure 3 and Tables 5 and 6). Even if the prognostic values of the three tumor groups were significantly different, the difference between leiomyosarcomas reclassified as undifferentiated pleomorphic sarcomas and tumors classified as leiomyosarcomas by both methods was lower than compared with tumors classified as undifferentiated pleomorphic sarcomas by both methods (subdistribution hazard ratio = 2.03; 95% CI: 1.33; 3.09; $P = 0.001$) (Table 6).

Could a Subclassification of Scoring Undifferentiated Pleomorphic Sarcomas Improve the Prognostic Value of the Smooth Muscle Differentiation Classification?

As described previously, the reclassified tumors represented an intermediate group in terms of metastatic risk between tumors classified as leiomyosarcomas or as undifferentiated pleomorphic sarcomas by both methods. We thus asked whether the reclassified group exhibited clinical differences as compared with the two other groups separately.

Prognosis of pleomorphic sarcomas
844
G Pérot *et al*

Table 2 Univariate analysis predicting metastatic outcome in 412 patients with sarcomas with complex genetics

	Total no. (no. of events)	Subdistribution hazard ratio (95% CI)	P-value (Gray)
Sex			
Male	197 (72)	Reference	0.6160
Female	215 (68)	0.92 (0.66; 1.28)	
Location			
Other	367 (115)	Reference	0.0017
Internal trunk	44 (24)	1.96 (1.29; 2.99)	
T3			
No	341 (99)	Reference	<0.0001
Yes	71 (41)	2.52 (1.75; 3.62)	
Deep			
No	49 (5)	Reference	0.0008
Yes	363 (135)	4.37 (1.84; 10.4)	
Grade			
I/II	145 (38)	Reference	0.0095
III	264 (100)	1.62 (1.13; 2.34)	
Vascular embolism			
No	224 (65)	Reference	<0.0001
Yes	27 (18)	3.84 (2.12; 6.93)	
Histological classification			
hUPS	222 (49)	Reference	<0.0001
hLMS	190 (91)	2.50 (1.77; 3.53)	
Smooth muscle differentiation classification			
sUPS	320 (85)	Reference	<0.0001
sLMS	92 (55)	2.62 (1.89; 3.62)	

Abbreviations: CI, confidence interval; Reference, indicates which parameter was taken as a reference for the analysis; hUPS, tumors histologically classified as undifferentiated pleomorphic sarcomas; hLMS, tumors histologically classified as leiomyosarcomas; sUPS, tumors classified as undifferentiated pleomorphic sarcomas by the smooth muscle differentiation classification; sLMS, tumors classified as leiomyosarcomas by the smooth muscle differentiation classification.

Table 3 Cumulative incidence of metastasis according to each classification method separately

	Cumulative incidence of metastasis	
Histological classif.	*hLMS (%)*	*hUPS (%)*
3 Years	39	21
6 Years	47	22
9 Years	50	23
	Cumulative incidence of metastasis	
SMD classif.	*sLMS (%)*	*sUPS (%)*
3 Years	44	24
6 Years	57	26
9 Years	61	28

Abbreviations: SMD, smooth muscle differentiation; hUPS, tumors histologically classified as undifferentiated pleomorphic sarcomas; hLMS, tumors histologically classified as leiomyosarcomas; sUPS; tumors classified as undifferentiated pleomorphic sarcomas by the smooth muscle differentiation classification; sLMS, tumors classified as leiomyosarcomas by the smooth muscle differentiation classification.
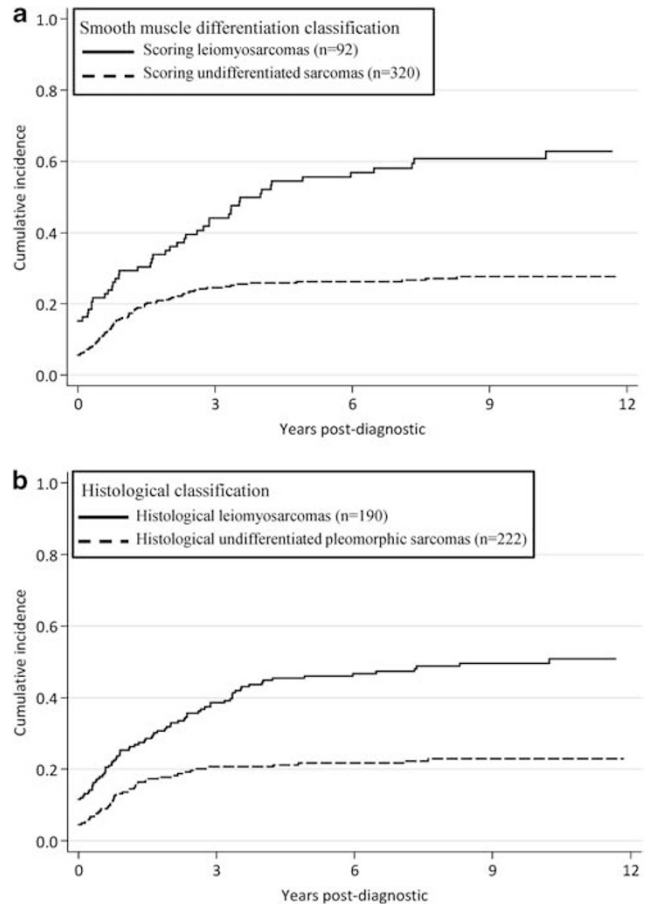

Figure 2 Metastatic outcome of 412 sarcomas with complex genetics classified according to two classification methods. (**a**) Cumulative incidence of metastasis using smooth muscle differentiation classification. (**b**) Cumulative incidence of metastasis of tumors classified using histological classification.

First, clinical parameters between reclassified tumors and tumors classified as leiomyosarcomas by both methods were compared (Supplementary Table S4). There were more male (χ^2 test, $P=0.0077$), more external trunk tumors (χ^2 test, $P<0.0001$) and high-grade tumors in the tumor group than in the other group (χ^2 test, $P<0.0001$). There were no significant differences between the two groups regarding tumor extension in the adjacent osseous and vasculonervous structures (T3), tumor depth, vascular embolism and patient age.

Secondly, clinical parameters between the reclassified tumors and the tumors classified as undifferentiated pleomorphic sarcomas by both methods were compared (Supplementary Table S5). There were slightly more tumors with an extension in the adjacent osseous and vasculonervous structures (T3) (χ^2 test, $P=0.0234$) and more grade III tumors (χ^2 test, $P=0.0008$) in the reclassified tumor group. Moreover in this group, patients were slightly younger than in the other tumor group (Student's *t*-test, $P=0.006$). No significant difference was observed between the two groups for other prognostic factors.

Table 4 Multivariate analysis predicting metastatic outcome in 412 patients with sarcomas with complex genetics

	P-value	sHR	97.5% CI
T3 (Ref = No)	0.00142	1.834	1.26; 2.66
Depth (Ref = No)	0.00190	3.786	1.63; 8.77
Grade (Ref = Grade I/II)	0.00428	1.704	1.18; 2.46
Histological classif. (Ref = hUPS)	0.04658	1.567	1.01; 2.44
SMD classification (Ref = sUPS)	0.00110	2.077	1.34; 3.22

Abbreviations: SMD, smooth muscle differentiation; sHR, subdistribution hazard ratio; CI, confidence interval; Ref, indicates which parameter was taken as a reference for the analysis; hUPS, tumors histologically classified as undifferentiated pleomorphic sarcomas; sUPS, tumors classified as undifferentiated pleomorphic sarcomas by the smooth muscle differentiation classification.
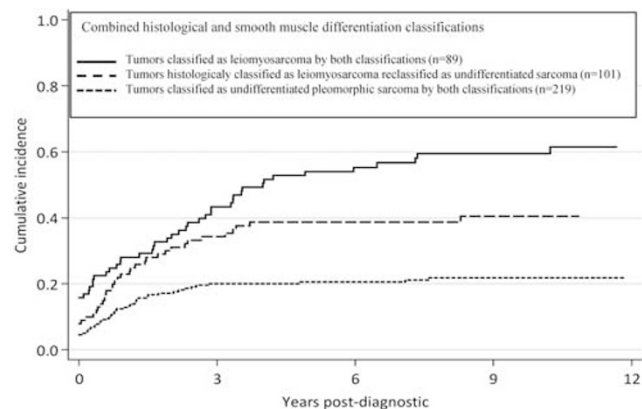
Table 6 Comparison of cumulative incidence of metastasis between non-reclassified and reclassified tumors

	Total no. (no. of events)	sHR (95% CI)	P-value (Fine and Gray)
hLMS→sUPS	101 (39)	Ref	0.0203
hsLMS	89 (52)	1.62 (1.08; 2.44)	
hsUPS	219 (46)	Ref	0.0010
hLMS→sUPS	101 (39)	2.03 (1.33; 3.09)	

Abbreviations: hsLMS, tumors classified as leiomyosarcomas by both classification methods; hsUPS, tumors classified as undifferentiated pleomorphic sarcomas by both classification methods; hLMS→sUPS, tumors histologically classified as leiomyosarcomas but reclassified as undifferentiated pleomorphic sarcomas by the smooth muscle differentiation classification; sHR, subdistribution hazard ratio; CI, confidence interval; Ref, criteria used as reference for the analysis.

Figure 3 Comparison between reclassified and non-reclassified tumors. Cumulative incidence of metastasis taking into account both classifications to classify each tumor.

Table 5 Cumulative incidence of metastasis of tumors according to a classification combining both methods

	Cumulative incidence of metastasis		
	hsLMS (%)	hLMS→sUPS (%)	hsUPS (%)
3 Years	43	34	20
6 Years	55	39	21
9 Years	59	40	22

Abbreviations: hsLMS, tumors classified as leiomyosarcomas by both classification methods; hsUPS, tumors classified as undifferentiated pleomorphic sarcomas by both classification methods; hLMS→sUPS, tumors histologically classified as leiomyosarcomas but reclassified as undifferentiated pleomorphic sarcomas by the smooth muscle differentiation classification.

As the smooth muscle differentiation score of the reclassified tumors tended to be higher than the score of tumors classified as undifferentiated pleomorphic sarcomas by both methods (Supplementary Table S6), subclassifications of scoring undifferentiated pleomorphic sarcomas according to different immunohistochemical criteria were established to test their prognostic values.

First the prognostic impact of a undifferentiated pleomorphic sarcomas' subclassification according to the number of positive immunohistochemical markers was tested. The undifferentiated pleomorphic sarcomas' group was thus subdivided into four subgroups: tumors with 0, 1, 2 and 3 positive immunohistochemical markers (Table 1). Tumors with 0, 1 or 3 positive immunohistochemical markers displayed a similar metastatic risk, whereas these groups presented a significantly lower metastatic risk as compared with scoring leiomyosarcomas ($P < 0.0001$, $P = 0.0012$ and $P = 0.0062$, respectively) (Supplementary Figure S1 and Supplementary Tables S7 and S8). The cumulative incidences of metastasis in the two positive marker group and in scoring leiomyosarcomas were not different (subdistribution hazard ratio = 1.68; 95% CI: 0.92; 3.07; $P = 0.0913$) (Supplementary Table S8). Indeed, this group of sarcomas with two positive markers exhibited an intermediate metastatic risk between the three positive marker group and the scoring leiomyosarcomas (with cumulative incidence of metastasis at 9 years: 33% for the three-marker group, 38% for the two-marker group and 61% for the scoring leiomyosarcomas group) (Supplementary Table S7).

The prognostic value of another subclassification of undifferentiated pleomorphic sarcomas was then tested. The undifferentiated pleomorphic sarcomas group was subdivided into three subgroups defined according to a score calculated by adding the immunohistochemical labeling intensities for the four markers. These three subgroups were: tumors with a score of 0, corresponding to tumors negative for all four markers; tumors with a score between 1 and 3; and tumors with a score between 4 and 6 (Supplementary Table S3). We observed that the metastatic risks of undifferentiated pleomorphic sarcomas with a score of 0 and tumors with a score between 1 and 3 were not significantly different (Figure 4 and Table 7). In the same way, the metastatic risks of tumors with a score between 4 and 6 and scoring leiomyosarcomas were close
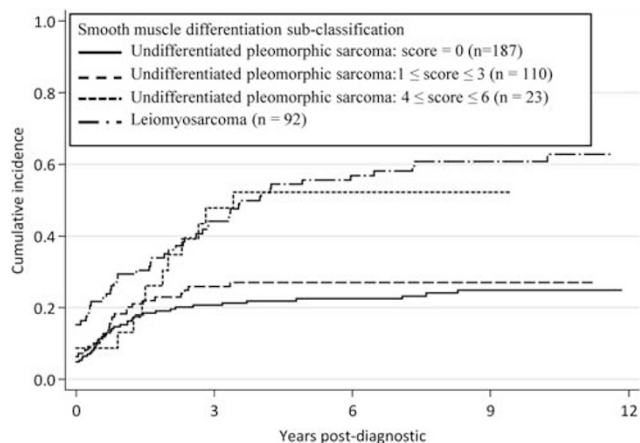
Figure 4 Comparison of metastatic outcome between tumors according to the immunohistochemical score.

Table 7 Cumulative incidence of metastasis of tumors according to their immunohistochemical score

	Cumulative incidence of metastasis			
	sUPS score = 0 (%)	*sUPS $1 \leq score \leq 3$ (%)*	*sUPS $4 \leq score \leq 6$ (%)*	*sLMS (%)*
3 Years	21	26	48	44
6 Years	22	27	52	57
9 Years	25	27	52	61

Abbreviations: sUPS, tumors classified as undifferentiated pleomorphic sarcomas by the smooth muscle differentiation classification; sLMS, tumors classified as leiomyosarcomas by the smooth muscle differentiation classification.

Table 8 Comparison of cumulative incidence of metastasis between non-reclassified and reclassified tumors

	Total no. (no. of events)	*sHR (95% CI)*	*P-value (Fine and Gray)*
sUPS: $0 \leq score \leq 3$	297 (74)	Ref	0.0048
sUPS: $4 \leq score \leq 6$	23 (12)	2.17 (1.27; 3.72)	
sUPS: $4 \leq score \leq 6$	23 (12)	Ref	0.5060
sLMS	92 (55)	1.23 (0.665; 2.28)	

Abbreviations: sHR, subdistribution hazard ratio; CI, confidence interval; Ref, criteria used as reference for the analysis; sUPS, tumors classified as undifferentiated pleomorphic sarcomas by the smooth muscle differentiation classification; sLMS, tumors classified as leiomyosarcomas by the smooth muscle differentiation classification.

(Figure 4 and Tables 7 and 8). Considering these results, we grouped together undifferentiated tumors with a score of 0 and those with a score between 1 and 3 and compared the cumulative incidence of metastasis between this new group and the group of tumors with a score between 4 and 6 (Table 8). A significant difference between the

cumulative incidences of these two groups was thus observed (subdistribution hazard ratio = 2.17; 95% CI: 1.27; 3.72; $P = 0.0048$) (Table 8). The tumor group with a score between 4 and 6, with an incomplete but strong smooth muscle differentiation, was thus significantly different to other undifferentiated pleomorphic sarcomas, and similar to the scoring leiomyosarcomas group in terms of metastatic risk (subdistribution hazard ratio = 1.23; 95% CI: 0.665; 2.28; $P = 0.5060$) (Table 8).

What is the Relationship between the Smooth Muscle Differentiation Classification and Tumor Biology?

As described previously, undifferentiated pleomorphic sarcomas with a score between 4 and 6 were closer to scoring leiomyosarcomas than to tumors with a score between 0 and 3 in terms of metastatic risk and we questioned whether they should be considered as a single entity or if they consisted of different entities, and if tumors with a score beween 4 and 6 displayed genomic features similar to those observed in scoring leiomyosarcomas.

For 78 tumors of this series, we disposed of array-CGH data so that we were able to carry genomic profile analysis. Pleomorphic sarcomas with complex genetics can be split into two groups according to their genomic profiles: a first group with few alterations mainly involving the full chromosome arm or entire chromosomal gain or loss ('complex/arm-type' genomic profile) and a second group with high level of chromosomal complexity ('complex/rearranged-type' genomic profile) (Table 9). The genomic profile comparison of tumors classified according to the histological classification has shown that in both tumor groups (histological leiomyosarcomas and histological undifferentiated pleomorphic sarcomas) the majority of tumors presented complex/rearranged genomic profiles (60% and 93% respectively) even if the distribution in these groups were significantly different (χ^2 test, $P = 0.0004$) (Table 9). When the profiles of tumors classified by the smooth muscle differentiation classification were compared, we observed that the vast majority of scoring leiomyosarcomas genomic profiles were 'complex/arm-type' genomic profiles (81%). In contrast, the genomic profile distribution in scoring undifferentiated pleomorphic sarcomas was similar to histological undifferentiated pleomorphic sarcomas one (7% of 'complex/arm-type' and 93% of 'complex/rearranged-type' genomic profiles for both groups) (Table 9). Profile distributions in the scoring undifferentiated pleomorphic sarcomas and scoring leiomyosarcomas groups were significantly different (Fisher's exact test, $P < 0.0001$) (Table 9).

To go further in the analysis, we observed that tumors classified as undifferentiated pleomorphic sarcomas or as leiomyosarcomas with both classifications exhibited significantly different genomic

Table 9 Tumors genomic profiles analysis

	'Complex/arm-type' genomic profile, N (%)	'Complex/rearranged-type' genomic profile, N (%)
hLMS (*n* = 35)	14 (40%)	21 (60%)
hUPS (*n* = 43)	3 (7%)	40 (93%)
sLMS (*n* = 16)	13 (81%)	3 (19%)
sUPS (*n* = 62)	4 (7%)	58 (93%)
hsLMS (*n* = 16)	13 (81%)	3 (19%)
hLMS→sUPS (*n* = 19)	1 (5%)	18 (95%)
hsUPS (*n* = 43)	3 (7%)	40 (93%)

Abbreviations: hLMS, tumors histologically classified as leiomyosarcomas; hUPS, tumors histologically classified as undifferentiated pleomorphic sarcomas; sLMS, tumors classified as leiomyosarcomas by the smooth muscle differentiation classification; sUPS, tumors classified as undifferentiated pleomorphic sarcomas by the smooth muscle differentiation classification; hsLMS, tumors classified as leiomyosarcomas by both classification methods; hLMS→sUPS, tumors histologically classified as leiomyosarcomas but reclassified as undifferentiated pleomorphic sarcomas by the smooth muscle differentiation classification; hsUPS, tumors classified as undifferentiated pleomorphic sarcomas by both classification methods.

profiles distribution, with more 'complex/rearranged-type' genomic profiles in the undifferentiated pleomorphic sarcomas group (93% of tumors *versus* 19% for leiomyosarcomas) (Fisher's exact test, $P < 0.0001$) (Table 9). Concerning leiomyosarcomas reclassified as undifferentiated pleomorphic sarcomas by the smooth muscle differentiation method, their genomic profile distribution was significantly different to the distribution of the tumors classified as leiomyosarcomas by both methods (χ^2 test, $P < 0.0001$): 5% *versus* 81% of 'complex/arm-type' genomic profiles and 95% *versus* 19% of 'complex/rearranged-type' genomic profiles, respectively (Table 9). Inversely, no significant difference was observed in terms of genomic profile distribution between the leiomyosarcomas reclassified as undifferentiated pleomorphic sarcomas by the smooth muscle differentiation method and the tumors classified as undifferentiated pleomorphic sarcomas by both methods (Fisher's exact test, $P = 0.80$) (Table 9). Moreover, all undifferentiated pleomorphic sarcomas with a score of 4 to 6 presented complex/rearranged genomic profiles such as tumors classified as undifferentiated pleomorphic sarcomas by both methods and thus seemed to differ from leiomyosarcomas (data not shown).

Discussion

Diagnosing sarcomas can be challenging, especially for specific subtypes. The clinical relevance of better identification of undifferentiated pleomorphic sarcomas has been previously shown, especially in cases of undifferentiated pleomorphic sarcomas with myogenic differentiation, which seem to be significantly more aggressive.[8,9]

In the present study, we assessed whether by taking into account the immunohistochemical labeling for four smooth muscle differentiation markers, we could propose a diagnostic and prognostic tool to classify sarcomas with complex genetics. According to this smooth muscle differentiation classification method, 25% of tumors were rediagnosed in another histotype. Among these reclassified tumors, 97% were initially histologically classified as leiomyosarcomas and then reclassified as undifferentiated pleomorphic sarcomas. Inversely, only 3% of tumors were reclassified from undifferentiated pleomorphic sarcoma group to leiomyosarcomas. These results suggested that the histological method of diagnosis may overestimate the incidence of the leiomyosarcoma histotype.

Among the studied proteins, transgelin has been recently shown as the best diagnostic marker in leiomyosarcomas *versus* all other sarcomas compared with SMA, desmin, h-CD and calponin.[14] However transgelin, such as SMA and calponin, is not exclusively expressed in smooth muscle tumors, so they cannot be considered specific to smooth myogenic differentiation.[8,15,16] In contrast, h-CD appeared specific, but lacked sensitivity.[14]

The smooth muscle differentiation classification method, taking into account not only positivity for smooth muscle markers but also their labeling intensity, exhibited the strongest prognostic value compared with other prognostic factors, including histological classification. These results show that it is of clinical relevance to use this classification to better assess the prognosis of sarcomas with complex genetics.

The cumulative incidence of metastasis in smooth muscle differentiation reclassified tumors was intermediate between tumors equally classified by both methods as undifferentiated pleomorphic sarcomas and as leiomyosarcomas, although it was closer to incidence for tumors classified as leiomyosarcomas by both methods. The comparison of clinical data has also shown significant differences between reclassified tumors and tumor equally classified by both methods as undifferentiated pleomorphic sarcomas or as leiomyosarcomas. According to these data, the reclassified tumors appear vastly heterogeneous. To test if a better scoring undifferentiated pleomorphic sarcomas classification might be defined in term of prognosis, this group of tumors was first subclassified according to the number of positive markers. However, the prognostic analysis has revealed that this classification was not efficient because tumors with two positive markers exhibited a higher metastatic risk than tumors with three positive markers. A more detailed study of these two groups revealed that both groups were in reality heterogeneous. Indeed, in each of these groups, there were both tumors with a labeling intensity of 1 for all positive markers, as well as tumors with at least one marker presenting an intensity of 2. In each group, establishing subgroups according to the labeling intensity (1 for all positive markers *versus* more than 1 for at least one of the positive markers) revealed that tumors with a higher labeling intensity

presented the worst prognosis (data not shown). In the same manner, the prognostic comparison of tumors with three positive markers and one in labeling intensity to those with two positive markers, but an intensity of more than 1 highlighted that the two positive marker tumors with higher labeling intensity were of worse prognosis. These indicate that it is not the number of positive markers alone which is relevant but that the labeling intensity must be taken into account.

For this reason, tumors were secondly classified using an immunohistochemical score defined by summing the labeling intensities obtained for all four markers for each tumor. The scoring undifferentiated pleomorphic sarcomas group could thus be subdivided into two groups: tumors with a score between 0 and 3, and tumors with a score ranging between 4 and 6. These two tumor groups exhibited significantly different metastatic risks.

To investigate whether there was a biological argument to consider undifferentiated pleomorphic sarcomas with a score between 4 and 6 as leiomyosarcomas or to consider them as a single entity, genomic profiles of 78 tumors for which we disposed of array-CGH data were analyzed. This analysis revealed that 81% of tumors classified as leiomyosarcomas by both methods presented alterations mainly involving the full chromosome arm or entire chromosomal gain or loss ('complex/arm-type' genomic profile), contrary to equally classified undifferentiated pleomorphic sarcomas that were characterized for the vast majority (93%) by very rearranged profiles ('complex/rearranged-type' genomic profile). Concerning leiomyosarcomas reclassified as undifferentiated pleomorphic sarcomas, they mostly presented rearranged profiles (95%), such as tumors classified as undifferentiated pleomorphic sarcomas by both classifications. Following the tumor reclassification, we could not only see that the leiomyosarcoma group was more homogeneous in terms of tumor genomic profiles but also that the reclassified tumors' genomic profile distribution was not significantly different to the distribution of the tumors classified as undifferentiated pleomorphic sarcomas by both methods. In addition, all undifferentiated pleomorphic sarcomas with a score between 4 and 6 presented rearranged profiles such as equally classified undifferentiated pleomorphic sarcomas, thus differing from equally classified leiomyosarcomas. These observations tend to show that tumors with a score between 4 and 6 were not leiomyosarcomas but undifferentiated pleomorphic sarcomas with a stronger myogenic differentiation and worse outcome.

Two groups of leiomyosarcomas have been previously described by Italiano et al.:[17] leiomyosarcomas of the extremities and leiomyosarcomas of the retroperitoneum.[17] The retroperitoneal leiomyosarcomas group was characterized by a higher risk of metastatic relapse and a distinct genomic and expression profile compared with other leiomyosarcomas.

Further, these tumors exhibited an upregulation of smooth muscle genes according to the expression data, results in line with those presented here. The authors also studied the genomic profiles of leiomyosarcomas and showed that 43% of leiomyosarcomas presented a 'complex/arm-type' profile and 57% a 'complex/rearranged-type' profile.[17] Moreover, a significant correlation was observed between the genomic profile and the tumor location, as 69% of tumors with an 'complex/arm-type' profile were internal trunk tumors, whereas 76% of 'complex/rearranged-type' tumors were located in the extremities. Concerning the association between the genomic profile type and tumor location, our results are consistent with these findings (a majority of internal trunk tumors in the 'complex/arm-type' group: 61% for scoring leiomyosarcomas and 57% for equally classified leiomyosarcomas, and a majority of tumors of the extremities in the 'complex/rearranged-type' group: 67% for scoring leiomyosarcomas and 90% for equally classified leiomyosarcomas). However, considering the distribution of 'arm/rearranged' profiles in leiomyosarcomas, our results were consistent with Italiano et al[17] for histological leiomyosarcomas only, and differed for equally classified leiomyosarcomas (43% versus 40% in histological leiomyosarcomas versus 81% in equally classified leiomyosarcomas). In our series, 95% of reclassified tumors presented a 'complex/rearranged-type' profile which could explain the enrichment of the scoring leiomyosarcomas group in tumors with 'complex/arm-type' profiles. In the Italiano et al[17] study, leiomyosarcomas were diagnosed according to histologic features that could explain this discrepancy.[17] According to our analysis, their group of leiomyosarcomas of the extremities could probably be in reality heterogeneous and could regroup both undifferentiated pleomorphic sarcomas and leiomyosarcomas. This heterogeneity could at least in part explain the two different biologies that the authors observed between their two leiomyosarcomas groups from different locations. To go further, it thus seems necessary to test if scoring leiomyosarcomas from retroperitoneum and from the extremities also correspond to two different biologies. It was not possible to perform expression profiles or genomic profiles comparisons in the present series as the scoring leiomyosarcomas group was too small to obtain statistically relevant results. However, Italiano et al.[17] also show that the retroperitoneal leiomyosarcomas group was characterized by a higher risk of metastatic relapse so we have compared metastastic outcome of tumors classified by histological classification or by smooth muscle differentiation according to tumor location. In our study, whatever the classification used, no significant association between location and outcome was observed (data not shown).

On the basis of our results, we thus propose a three-group classification of pleomorphic sarcomas with complex genetics, as summarized in Figure 5.

Prognosis of pleomorphic sarcomas
G Pérot *et al*
849

We propose immunohistochemistry experiments for the four markers to first distinguish between leiomyosarcomas and undifferentiated pleomorphic sarcomas: tumors with a complete smooth muscle differentiation phenotype characterized by positivity for all four markers should be considered as leiomyosarcomas, and all other tumors as undifferentiated pleomorphic sarcomas. Then, for the undifferentiated pleomorphic sarcomas group, the labeling intensities must be defined and added to establish the smooth muscle differentiation score: a tumor with a score of 0 to 3 should be considered as an undifferentiated pleomorphic sarcomas of best prognosis, and a tumor with a score of 4 to 6 should be considered as an undifferentiated pleomorphic sarcomas with an intermediate smooth muscle differentiation and a worse prognosis (Figure 5).

In a previous publication, we have shown that the *MYOCD* gene, a transcriptional cofactor of the serum response factor (SRF) regulating smooth muscle development and differentiation,[18] was overexpressed in 74% of leiomyosarcomas and amplified overexpressed in 53% of leiomyosarcomas, essentially in retroperitoneal leiomyosarcomas (80%).[11] This series is in line with our previous report. Indeed, the *MYOCD* gene was overexpressed in 58% of leiomyosarcomas in the present study (14/24), among which 71% were retroperitoneal leiomyosarcomas (10/14) (data not shown). In this previous publication, forced *MYOCD* expression in undifferentiated sarcoma cell lines has been shown as conferring a strong smooth muscle differentiation phenotype by inducing the expression of calponin, transgelin, SMA, h-CD and increasing cell migration abilities.[11] In the present study, the tumor strong smooth muscle differentiation is related to metastasis. These results are also in line with those previously published, which have shown that muscle differentiation pathways are over-represented in metastatic leiomyosarcomas *versus* non-metastatic leiomyosarcomas, reflecting the implication of smooth muscle differentiation in the metastasis process.[17] However, the role of smooth muscle proteins in tumor oncogenesis and invasion is still controversial. h-CD and calponin were described as tumor suppressor genes and migration inhibitors,[19,20] while transgelin seemed to be a potent inducer of tumor invasion[21] such as the LPP protein,[22–24] which is overexpressed in leiomyosarcomas and whose expression was induced by *MYOCD* overexpression.[11] Likewise *MYOCD* and myocardin-related transcription factors were also supposed to be tumor suppressor genes.[18,25] However, in some cellular models such as breast carcinoma cell lines or mesenchymal stem cells, they have been described as cell migration enhancers that do not interfere with cell proliferation.[26,27] The hypothesis that the impact of smooth muscle differentiation on diverse cellular processes could be distinct according to the phosphorylation status of proteins, the balance involved between various proteins with opposite functions, the proteins interactions implicated and the activated pathway could be made. The cellular context thus appears critical for smooth muscle proteins functions. Because of the great complexity of sarcomas, only functional studies in sarcoma cell lines or murine models could really provide answers to these questions in the sarcoma context. A precise assessment of the biological pathways implicated in smooth muscle sarcomas seems necessary.

In conclusion, our results show that the smooth muscle differentiation classification method (Figure 5) could not only be a useful diagnostic tool but also a relevant prognostic tool for undifferentiated pleomorphic sarcomas. We demonstrate here that it is of clinical relevance to assess the immunohistochemical score for the four markers for any suspected leiomyosarcomas or undifferentiated pleomorphic sarcomas.
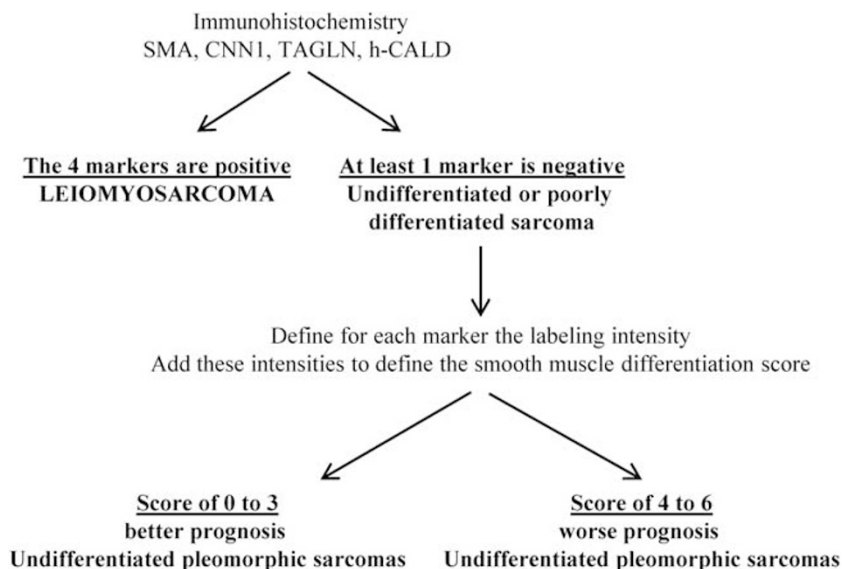

Figure 5 Smooth muscle differentiation classification summary.

Acknowledgments

Disclosure/conflict of interest

The authors declare no conflict of interest.

References

1 Fletcher CDM, Gronchi A, Singer S, *et al.* Tumours of soft tissue: introduction, In: Fletcher CDM, Bridge JA, Hogendoorn PCW, Mertens F (eds). World Health Organisation Classification of Tumours of Soft Tissue and Bone. IARC Press: Lyon, France; 2013, pp 13–18.

2 Coindre JM, Pedeutour F, Aurias A. Well-differentiated and dedifferentiated liposarcomas. Virchows Arch 2010;456:167–179.

3 Guillou L, Aurias A. Soft tissue sarcomas with complex genomic profiles. Virchows Arch 2010;456:201–217.

4 Chibon F, Mariani O, Derré J, *et al.* A subgroup of malignant fibrous histiocytomas is associated with genetic changes similar to those of well-differentiated liposarcomas. Cancer Genet Cytogenet 2002;139:24–29.

5 Chibon F, Mariani O, Mairal A, *et al.* The use of clustering software for the classification of comparative genomic hybridization data. An analysis of 109 malignant fibrous histiocytomas. Cancer Genet Cytogenet 2003;141:75–78.

6 Lazar A, Evans HL, Shipley J. Leiomyosarcoma, In: Fletcher CDM, Bridge JA, Hogendoorn PCW, Mertens F (eds). World Health Organisation Classification of Tumours of Soft Tissue and Bone. IARC Press: Lyon, France; 2013, pp 111–115.

7 Fletcher CDM, Chibon F, Mertens F. Undifferentiated/ unclassified sarcoma, In: Fletcher CDM, Bridge JA, Hogendoorn PCW, Mertens F (eds). World Health Organisation Classification of Tumours of Soft Tissue and Bone. IARC Press: Lyon, France; 2013, pp 236–238.

8 Fletcher CD, Gustafson P, Rydholm A, *et al.* Clinico-pathologic re-evaluation of 100 malignant fibrous histiocytomas: prognostic relevance of subclassification. J Clin Oncol 2001;19:3045–3050.

9 Deyrup AT, Haydon RC, Huo D, *et al.* Myoid differentiation and prognosis in adult pleomorphic sarcomas of the extremity: an analysis of 92 cases. Cancer 2003;98:805–813.

10 Mills AM, Beck AH, Montgomery KD, *et al.* Expression of subtype-specific group 1 leiomyosarcoma markers in a wide variety of sarcomas by gene expression analysis and immunohistochemistry. Am J Surg Pathol 2011;35:583–589.

11 Pérot G, Derré J, Coindre JM, *et al.* Strong smooth muscle differentiation is dependent on myocardin gene amplification in most human retroperitoneal leiomyosarcomas. Cancer Res 2009;69:2269–2278.

12 Fine JP, Gray RJ. A proportional hazards model for the subdistribution of a competing risk. J Am Stat Assoc 1999;94:496–509.

13 Chibon F, Lagarde P, Salas S, *et al.* Validated prediction of clinical outcome in sarcomas and multiple types of cancer on the basis of a gene expression signature related to genome complexity. Nat Med 2010;16: 781–787.

14 Robin YM, Penel N, Pérot G, *et al.* Transgelin is a novel marker of smooth muscle differentiation that improves diagnostic accuracy of leiomyosarcomas: a comparative immunohistochemical reappraisal of myogenic markers in 900 soft tissue tumors. Mod Pathol 2013;26: 502–510.

15 Ono H, Yoshikawa H, Ueda T, *et al.* Expression of smooth muscle calponin in synovial sarcoma. Sarcoma 1999;3:107–113.

16 Fisher C, Montgomery E, Healy V. Calponin and h-caldesmon expression in synovial sarcoma; the use of calponin in diagnosis. Histopathology 2003;42:588–593.

17 Italiano A, Lagarde P, Brulard C, *et al.* Genetic profiling identifies two classes of soft-tissue leiomyosarcomas with distinct clinical characteristics. Clin Cancer Res 2013;19:1190–1196.

18 Pipes GC, Creemers EE, Olson EN. The myocardin family of transcriptional coactivators: versatile regulators of cell growth, migration, and myogenesis. Genes Dev 2006;20:1545–1556.

19 Mayanagi T, Sobue K. Diversification of caldesmon-linked actin cytoskeleton in cell motility. Cell Adh Migr 2011;5:150–159.

20 Horiuchi A, Nikaido T, Taniguchi S, *et al.* Possible role of calponin h1 as a tumor suppressor in human uterine leiomyosarcoma. J Natl Cancer Inst 1999;91: 790–796.

21 Lee EK, Han GY, Park HW, *et al.* Transgelin promotes migration and invasion of cancer stem cells. J Proteome Res 2010;9:5108–5117.

22 Grunewald TG, Pasedag SM, Butt E. Cell adhesion and transcriptional activity—defining the role of the novel protooncogene LPP. Transl Oncol 2009;2:107–116.

23 Majesky MW. Organizing motility: LIM domains, LPP, and smooth muscle migration. Circ Res 2006;98: 306–308.

24 Petit MM, Lindskog H, Larsson E, *et al.* Smooth muscle expression of lipoma preferred partner is mediated by an alternative intronic promoter that is regulated by serum response factor/myocardin. Circ Res 2008;103: 61–69.

25 Milyavsky M, Shats I, Cholostoy A, *et al.* Inactivation of myocardin and p16 during malignant transformation contributes to a differentiation defect. Cancer Cell 2007;11:133–146.

26 Madonna R, Wu D, Wassler M, *et al.* Myocardin-A enhances expression of promyogenic genes without depressing telomerase activity in adipose tissue-derived mesenchymal stem cells. Int J Cardiol 2012;159.

27 Medjkane S, Perez-Sanchez C, Gaggioli C, *et al.* Myocardin-related transcription factors and SRF are required for cytoskeletal dynamics and experimental metastasis. Nat Cell Biol 2009;11:257–268.

Supplementary Information accompanies the paper on Modern Pathology website (http://www.nature.com/modpathol)