Imaging, Diagnosis, Prognosis

Clinical Cancer Research

Genetic Profiling Identifies Two Classes of Soft-Tissue Leiomyosarcomas with Distinct Clinical Characteristics

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Abstract

Purpose: Data about the prognostic factors of soft-tissue leiomyosarcomas and their correlation with molecular profile are limited.

Experimental Design: From 1990 to 2010, 586 adult patients with a primary soft-tissue leiomyosarcoma were included in the French Sarcoma Group (GSF) database after surgery of the primary tumor. Multivariate analyses were conducted by Cox regression model in a backward stepwise procedure. Genetic profiling was conducted for 73 cases.

Results: Median age was 59 years (range, 21–98 years). The median follow-up of patients alive was 46 months. The 5-year metastasis-free survival (MFS) rate was 51% (95% location and grade > I were independent adverse prognostic factors for MFS). The 5-year overall survival (OS) rate was 63% [95% confidence interval (CI), 59–67]. On multivariate analysis, age $\geq$ 60 years old, tumor size > 5 cm, deep location, and grade > I were independent adverse prognostic factors for OS. Molecular profiling identified specific clusters with activation of different biologic pathways: retroperitoneal leiomyosarcomas are characterized by overexpression of genes involved in muscle differentiation and nonretroperitoneal leiomyosarcomas characterized by overexpression of genes mainly involved in extracellular matrix, wounding, and adhesion pathways. The CINSARC signature but not comparative genomic hybridization (CGH) profiling was predictive of outcome.

Conclusion: Soft-tissue leiomyosarcomas represent a heterogeneous group of tumors with at least two categories, retroperitoneal and extremities leiomyosarcomas, having specific clinical outcome and molecular features. Future clinical trials should consider this heterogeneity for a better stratification of patients. *Clin Cancer Res; 19(5); 1190–6. ©2013 AACR.*

Introduction

Leiomyosarcoma are an uncommon group of malignant tumors composed of cells showing distinct smooth-muscle differentiation (1). These tumors occur mainly in adults in any location of the body (soft-tissue or viscera). Soft-tissue leiomyosarcomas represent 10% to 15% of all soft-tissue sarcomas. The most frequent locations are the limbs followed by the retroperitoneum. Data related to the clinical outcome of soft-tissue leiomyosarcomas are mainly limited to small, single-institution, nonexhaustive, or out-of-date series (2–7). Moreover, only few data about the molecular characteristics of leiomyosarcomas are available. Most of such studies analyzed a small number of cases and/or mixed visceral and soft-tissue and primary and metastatic specimens (8–18). The main objective of our study was to investigate the clinical outcome, the prognostic factors of soft-tissue leiomyosarcomas and the correlation between molecular profiles and clinical characteristics.

Material and Methods

Patients

From 1990 to 2010, 586 adult patients ($\geq$18 years old) with a nonmetastatic soft-tissue leiomyosarcomas underwent surgery of the primary tumor and were included in the

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

Leiomyosarcomas represent one of the most frequent sarcoma subtypes and can occur in the soft-tissue compartment or visceral sites. This study focused on soft-tissue leiomyosarcomas to identify their prognostic factors and their molecular characteristics. Our results showed that soft-tissue leiomyosarcomas were a heterogeneous group of tumors with at least two categories, retroperitoneal and peripheral leiomyosarcomas, having peculiar clinical and molecular features.

French Sarcoma Group (GSF) database. All the cases were reviewed by the members of the pathologic subcommittee of the GSF. The histologic diagnosis was established according to the World Health Organization Classification of Tumors (1). The histologic grade was determined after central review as previously described according to the Fédération Nationale des Centres de Lutte Contre le Cancer (FNCLCC) grading system (19, 20).

Selection of cases for genetic profiling

Genetic profiling included array comparative genomic hybridization (CGH) and gene expression array. The selection of cases was based on the following inclusion criteria: availability of frozen tumor material from the primary tumor, absence of chemotherapy or radiotherapy given before tumor sampling, and patient consent. Seventy-three cases followed these criteria. Their characteristics (Supplementary Table S1) were similar to that of the entire cohort except for the proportion of small tumors (<5 cm) which was significantly lower in the molecular cohort (9.5% vs. 36%, $P = 0.03$) as the result of the obvious lower probability to collect frozen material from small samples.

Array CGH

gDNA was isolated using a standard phenol–chloroform extraction protocol. Array-based CGH experiments were done with a DNA microarray developed in our laboratory. A total of 3,874 BAC/PAC DNAs (BACPAC Resources Center, Children's Hospital, Oakland Research Institute, Oakland, CA) were spotted in triplicate on UltraGAPS slides (Corning). These clones cover the whole genome with a resolution of 1 Mb. The probes were prepared and hybridized as previously described (21). The data were analyzed with software developed at Institut Curie (CAPweb, http://bioinfo-out.curie.fr/CAPweb/). Cyanine-5/cyanine-3 ratios >2 were considered as amplifications, and ratios >1.2 and <0.8 were considered as gains and losses, respectively. Analysis of array CGH (computation of genomic alterations) was provided by the VAMP interface (http://bioinfo.curie.fr/vamp; ref. 22).

Gene expression profile

Total RNAs were extracted from frozen tumor samples with TRIzol reagent (Life Technologies, Inc.) and purified using the RNeasy Min EluteTM Cleanup Kit (Qiagen) according to the manufacturer's procedures. We checked RNA quality on an Agilent 2100 bioanalyzer (Agilent Technologies). Samples were then analyzed on Human Genome U133 Plus 2.0 array (Affymetrix), according to the manufacturer's procedures (GEO access number: GSE21050). We simultaneously normalized all microarray data using the GCRMA algorithm (23). The *t* tests were conducted using Genespring (Agilent Technologies), and *P* values were adjusted using the Benjamini–Hochberg procedure. The *P* value and fold change cut-off for gene selection were 0.001 and 3, respectively. Gene ontology (GO) analysis was conducted to establish statistical enrichment in GO terms using Genespring (Agilent Technologies).

Statistical analysis

The statistical analysis of baseline demographics and clinical outcome are based on all data available up to the cutoff date of July 31, 2011. Descriptive statistics were used to show the distribution of variables in the population. Overall survival (OS) was defined as the interval between histologic diagnosis and the time of death or last follow-up. Metastasis-free survival (MFS) was defined as the interval between histologic diagnosis and the time of distant recurrence or the last follow-up. Patients who did not develop metastasis (for MFS) or remained alive (for OS) at final follow-up were censored at that time. Follow-up times were described as median by use of the inverse Kaplan–Meier estimator (24). Survival rates were estimated with the use of the Kaplan–Meier method and compared using the log-rank test. Multivariate analyses were conducted by Cox regression model in a backward stepwise procedure. Univariate and multivariate analyses included the following variables: age, sex, anatomic site, tumor size, tumor location (superficial or deep), and FNCLCC grade. Variables associated with survival with a $P < 0.05$ in the univariate analysis were included in the multivariate regression. Analyses were carried out using SAS 19.0 statistical software. All statistical tests were 2-sided, and $P < 0.05$ indicated statistical significance.

Results

Patients

The patients' characteristics are described in Table 1. Median age was 59 years (range, 21–98 years). The majority of patients had a leiomyosarcoma of the extremities (62.5%), larger than 5 cm (59%), and deeply located (79%). About 12% of patients had grade I disease, 36% had grade II, and 47% had grade III. Grading was missing in 5% of cases. Three hundred and seven patients (52%) received adjuvant radiotherapy. One hundred and nine patients (18%) received adjuvant chemotherapy. In all the cases, doxorubicin was delivered either alone or in combination with other drugs (dacarbazine with or without cyclophosphamide and vincristine: CYVADIC protocol, or ifosfamide with or without dacarbazine and mesna: AI or MAID: protocols). The factors significantly associated with a

Table 1. Patient characteristics ($N = 586$)

	No. of patients (%)
Age, y	
Median	59
Range	21–98
Sex	
Male	253 (43.0)
Female	333 (57.0)
Tumor site	
Head and neck	23 (4.0)
Internal trunk	131 (22.5)
Limb	366 (62.5)
Trunk wall	66 (11)
Tumor location	
Deep	462 (79)
Superficial	116 (20)
Unknown	8 (1)
Tumor size, cm	
<5	212 (36)
≥5	345 (59)
Unknown	29 (5)
Grade	
I	69 (12)
II	210 (36)
III	279 (47)
Unknown	28 (5)
Chemotherapy	
Adjuvant	109 (18)
Neoadjuvant	88 (15)
No	385 (66)
Unknown	4 (1.0)
Radiotherapy	
Adjuvant	307 (52.0)
Neoadjuvant	8 (1.5)
No	263 (45.0)
Unknown	8 (1.5)

higher likelihood to receive adjuvant chemotherapy were: age < 60 years (25.5% vs. 11.5%, $P < 0.0001$), deep location of the tumor (21% vs. 9.5%, $P = 0.005$), and grade III disease (30% vs. 10.5% for grade II vs. 1.5% for grade I, $P < 0.0001$).

Prognostic factors

Metastasis-free survival. The median follow-up of patients alive was 46 months. At the time of analysis, 246 patients (42%) had metastatic recurrence. The median MFS was 82 months [95% confidence (CI), 46–118; Supplementary Fig. S1). The 1-, 5-, and 10-year MFS rates were 83% (95% CI, 80–86), 51% (95% CI, 47–55), and 45% (95% CI, 41–49), respectively (Supplementary Fig. S1). On multivariate analysis (Supplementary Table S2 and table 2), retroperitoneal location, tumor size > 5 cm, deep location, and grade > I were independent adverse prognostic factors for MFS. The most significant adverse prognostic factor for MFS was grade III (HR, 3.5; 95% CI, 1.7–7.4; $P = 0.001$; Supplementary Fig. S2).

Overall survival. At the time of analysis, 209 patients (35%) had died and 377 (65%) were still alive. One hundred and sixty-four deaths (78%) were the result of sarcoma (including 2 deaths related to the treatment) and 45 (22%) the result of other causes. The median OS was 116 months (95% CI, 92–140 months). The 1-, 5-, and 10-year OS rates were 95% (95% CI, 93–97), 63% (95% CI, 59–67), and 49% (95% CI, 45–53), respectively (Supplementary Fig. S1). On multivariate analysis (Supplementary Table S2 and Table 2), age ≥ 60 years old, tumor size > 5 cm, deep location, and grade > I were independent adverse prognostic factors for OS. As for MFS, the most significant adverse prognostic factor for OS was grade III (HR, 6.2; 95% CI, 1.9–19.8; $P = 0.001$; Supplementary Fig. S3).

Genomic profiling

Genomic profiling was conducted on 73 leiomyosarcomas and except for 5 cases which presented a flat profile, we observed for 68 leiomyosarcomas, a characteristic complex profile with the most frequent alterations being losses of chromosomes 10q, 13q, 16q and 17p, and gains of 17p (Fig. 1). According to both the number and the type of alterations, we identified 2 types of recurrent profiles (Fig. 1). A first group of 29 tumors (43%) had few alterations (<30) mainly involving the full chromosome arm or entire chromosomal gain or loss. We called this group the "arm" profile group. A second group of 39 tumors (57%) was characterized by a high level of chromosomal complexity with more than 30 alterations. We called this group the "rearranged" profile. We identified a significant correlation between the genomic profile and the tumor location as 69% of tumors of the "arm" profile group were retroperitoneal, whereas 76% of the tumors of the "rearranged" profile group were located in the extremities ($P = 0.02$). However, on univariate analysis, the genomic profile ("arm" vs. "rearranged") was not predictive of MFS ($P = 0.18$; data not shown).

Expression profiling

Gene expression profiles of the 73 leiomyosarcomas were re-examined to test the hypothesis that gene expression in the tumor is associated to genome profile, tumor location, or metastatic outcome. We thus conducted 3 *t* tests to compare the expression profiles of tumors classified according to (i) genomic profile type (arm vs. rearranged); (ii) tumor location (retroperitoneal vs. extremities); and (iii) metastatic outcome (metastasis vs. nonmetastasis). We identified 445 genes that were upregulated [fold change (FC) > 3; $P > 0.001$] in the "arm" profile group in comparison with the "rearranged" profile and 423 genes that were upregulated (FC > 3; $P > 0.001$) in leiomyosarcomas located in the internal trunk in comparison with leiomyosarcomas located in the extremities. As expected, most of the differentially expressed genes are common to both comparisons (Fig. 2A) and the pathways overrepresented were extremely similar in both groups and were mainly involved in muscle differentiation (Supplementary Table S3). We also found

Table 2. Significant prognostic factors for MFS and OS (multivariate analysis)

	MFS		OS	
Variable	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
Age, y				
<60	— (—)	NS	1.0 (—)	<0.0001
≥60			1.7 (1.3–2.3)	
Tumor site				
Limb	1.0 (—)	0.01	— (—)	NS
Trunk wall	0.6 (0.3–1)			
Head and neck	1.0 (0.4–2.4)			
Internal trunk	1.4 (1.1–2)			
Tumor size, cm				
<5	1.0 (—)	0.04	1.0 (—)	0.007
≥5	1.4 (1.1–2)		1.7 (1.1–2.5)	
Tumor location				
Superficial	1.0 (—)	0.001	1.0 (—)	0.02
Deep	2.6 (1.5–4.7)		2.0 (1.1–3.5)	
FNCLCC grade				
I	1.0 (—)	0.001	1.0 (—)	0.001
II	2.5 (1.2–5.2)		4.2 (1.3–13.5)	
III	3.5 (1.7–7.4)		6.2 (1.9–19.8)	

that the *MYOCD* gene (17p12 chromosomal region) was the most overexpressed in leiomyosarcomas of the internal trunk as compared with leiomyosarcomas of the extremities (absolute FC = 100.2). As *MYOCD* was previously reported as amplified in a subset of leiomyosarcomas, we have assessed the genomic status of this gene in our series. We found a high-level amplification and a gain of the *MYOCD* gene in 7 and 17 cases, respectively. Amplification of the *MYOCD* gene was significantly associated with high expression ($P < 0.0001$). Moreover, we identified 248 and 156

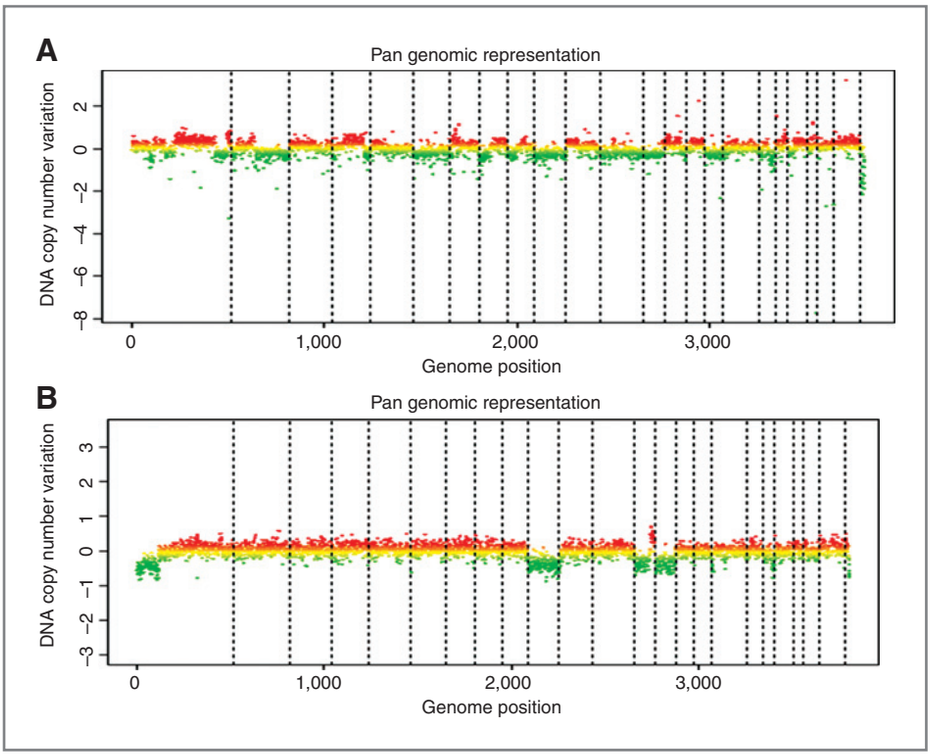

Figure 1. Genomic profiles (CGH) one case of leiomyosarcoma (LMS) of extremity with a "rearranged profile" (A) and one case of LMS of internal trunk with an "arm" profile (B).

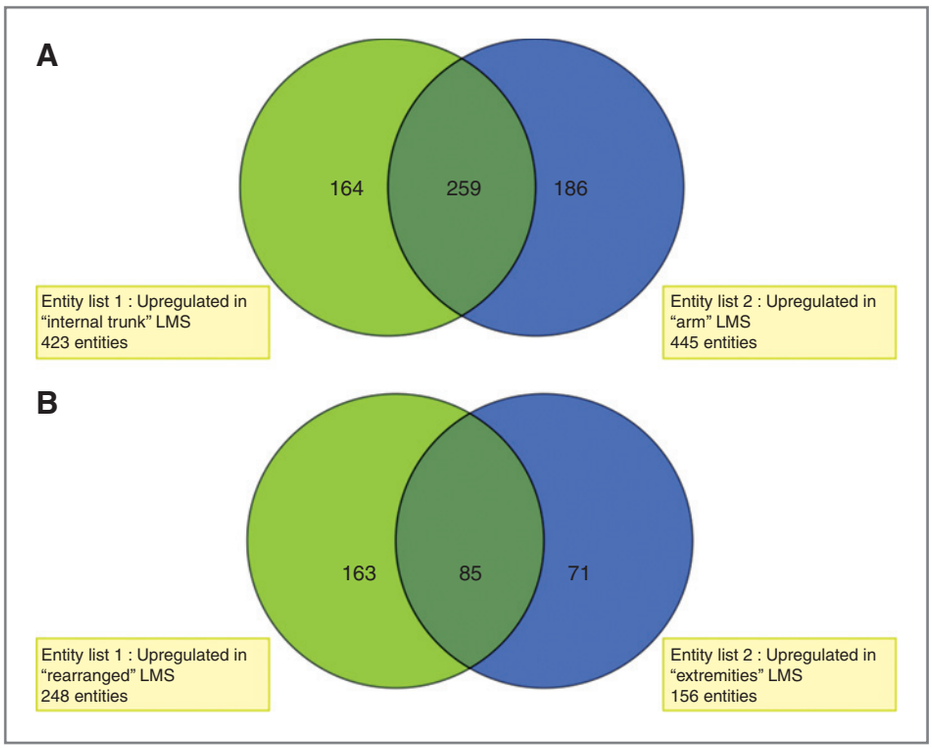

Figure 2. Upregulated genes in internal trunk leiomyosarcomas (LMS) and "arm profile" LMS (A) and extremities LMS and "rearranged profile" LMS (B).

genes that were upregulated in the "rearranged" profile group in comparison with the "arm" profile group and in leiomyosarcomas of the extremities in comparison with retroperitoneal leiomyosarcomas. The majority of these genes were common to both comparisons (Fig. 2B) and encoded proteins involved mainly in extracellular matrix, wounding, and adhesion pathways (Supplementary Table S4). On the contrary, no common gene or pathway were observed between "Retroperitoneal" and "Rearranged" leiomyosarcomas on one hand and between "Extremities" and "Arm" LMS on the other hand (data not shown).

Regarding metastasis outcome, few genes were significantly differentially expressed between leiomyosarcomas with or without metastasis (18 up- and 9 downregulated in metastatic cases, FC > 2; $P < 0.05$). Of note, upregulated genes are involved in muscle differentiation and downregulated ones in lipids metabolism (Supplementary Table S5). This signature failed to predict significantly metastatic outcome (data not shown), we thus tested a previously published signature, that is, CINSARC, and survival analysis (Fig. 3) revealed that the CINSARC classification split the tumors into 2 groups with very different MFS ($P = 5.8 \times 10^{-5}$).

Discussion

We report here the first large series investigating the prognostic factors and the molecular profile of soft-tissue leiomyosarcomas.

The 5-year OS (63%) rate was similar to that reported by Svarvar and colleagues in a series of 206 patients with localized leiomyosarcomas (7). Of note, in this series, the 5-year MFS was higher than in our study (74% vs. 51%). This result is probably explained by the exclusion of retroperitoneal leiomyosarcomas in the study of Svarvar and colleagues, leiomyosarcomas in this location being characterized by an higher risk of metastatic relapse as we have shown here. Although the majority or metastatic recurrence (64%) occurred within 2 years after the initial diagnosis, a

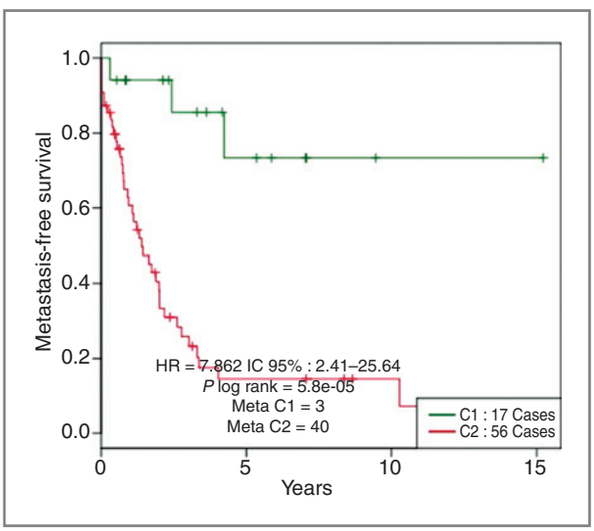

Figure 3. Kaplan–Meier analysis of MFS of 73 leiomyosarcomas (LMS) according to the CINSARC category (C1: patients with low expression of CINSARC genes; C2: patients with high expression of CINSARC genes).

significant proportion of patients experienced late treatment failure up to 11 years after initial diagnosis. This underscores the need for a prolonged follow-up of patients with primary resected leiomyosarcomas. Interestingly, patients with metastatic recurrence occurring >2 years after the initial diagnosis had a significantly better outcome than patients who relapsed earlier (data not shown). We and others have previously reported such a correlation between a longer time to recurrence and a better postrecurrence survival in soft-tissue and bone sarcomas (25–27). Nevertheless, this finding should be interpreted with caution. Indeed, the design of our study did not allow us to analyze the impact on postrecurrence survival of several key variables such as the type of management of metastatic recurrence and particularly the role of resection of metastases or the role of an additional lines of palliative chemotherapy in patients already treated with chemotherapy in the adjuvant setting.

The large cohort included in our study as well as the mature follow-up allowed us to identify robust prognostic factors for patients with localized leiomyosarcomas. In our series, grade III, retroperitoneal tumor site, deep location, and tumor size > 5 cm were independent predictors of poor MFS. These findings were consistent with the data from smaller series which have already shown a significant correlation of grade, tumor depth, and tumor size with the risk of metastatic relapse (3, 5–7). Previous series focusing on retroperitoneal sarcomas have already shown the higher metastatic risk of leiomyosarcomas in comparison with other retroperitoneal histologic subtypes including liposarcomas (28, 29). Our study shows that retroperitoneal leiomyosarcomas represent among soft-tissue leiomyosarcomas, a specific clinical and molecular entity. Indeed, in comparison with leiomyosarcomas of the extremities, retroperitoneal leiomyosarcomas are characterized by a higher risk of metastatic relapse and a distinct genomic and expression profile. Most of the genes overexpressed in retroperitoneal leiomyosarcomas encode proteins involved in muscle differentiation. Nonretroperitoneal leiomyosarcomas are on the contrary characterized by overexpression of genes encoding proteins mainly involved in extracellular matrix, wounding, and adhesion pathways. The capacity of molecular profiling to identify leiomyosarcoma clusters was previously suggested by a study from Beck and colleagues analyzing a limited series of cases and showing that leiomyosarcomas include distinct molecular subtypes including one characterized by an overexpression of muscle-associated genes (18). However, in the study of Beck and colleagues, 26 of the 52 samples were not primary but metastatic samples with potential changes in gene expression patterns in comparison to the primary tumor and only 6 retroperitoneal cases were included, precluding any possible correlation with clinical characteristics or outcome. Interestingly, a recent study has shown that retroperitoneal leiomyosarcomas carry a frequent amplification of the *MYOCD* gene which is also the most differentially expressed gene between leiomyosarcomas and retroperitoneal undifferentiated sarcomas (30). *MYOCD* is involved in smooth-muscle differentiation and in the regulation of cell migration (31, 32). Its inactivation has been shown to reduce not only smooth-muscle differentiation gene expression but also cell migration in leiomyosarcoma cell lines, suggesting a potential role in metastatic progression.

In this regard, we have observed that muscle differentiation pathways are overrepresented in metastatic cases versus nonmetastatic cases, reflecting the high metastatic potential of retroperitoneal leiomyosarcomas which are often well differentiated. However, the simple comparison of expression profiling of patients with leiomyosarcomas with and without metastases did not allow us to identify a specific prognostic molecular signature for leiomyosarcomas. We recently published a 67-gene expression prognostic signature related to genome complexity (CINSARC for Complexity INdex in SARComas) which predict outcome in sarcomas with complex genomics such as leiomyosarcomas (33). As expected, this signature was able to predict outcome in the present series of soft-tissue leiomyosarcomas. Further investigations are needed to investigate how this molecular signature can help to identify patients who are more likely to benefit of adjuvant treatments such as chemotherapy to prevent metastatic relapse.

Clinicians involved in the management of soft-tissue sarcomas are well aware of the high heterogeneity of this group of rare malignancies including more than 50 histologic subtypes. By focusing our investigations on soft-tissue leiomyosarcomas, we were able to clarify the prognostic factors of leiomyosarcomas and to identify even more heterogeneity with at least 2 categories retroperitoneal and peripheral leiomyosarcomas having peculiar clinical and molecular features. The next step of our work will be to identify "druggable" specific molecular aberrations in these specific leiomyosarcoma categories.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: A. Italiano, J.-Y. Blay, F. Chibon
Development of methodology: A. Italiano, F. Chibon
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Italiano, P. Largarde, P. Terrier, M. Laë, B. Marques, D. Ranchere-Vince, J.-J. Michels, M. Trassard, A. Cioffi, S. Piperno-Neumann, C. Chevreau, J.-Y. Blay, C. Delcambre, N. Isambert, N. Penel, J.-O. Bay, S. Bonvalot, J.M. Coindre, F. Chibon
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Italiano, C. Brulard, P. Terrier, S. Bonvalot, A. Le Cesne
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