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Expression of subtype-specific Group 1 leiomyosarcoma markers in a wide variety of sarcomas by gene expression analysis and immunohistochemistry

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Abstract

Leiomyosarcomas (LMS) constitute approximately one quarter of all sarcomas and are usually defined by morphologic criteria and/or immunoreactivity for actin or desmin. Among high grade lesions, the distinction from undifferentiated pleomorphic sarcoma (UPS) can be problematic and previous studies have shown that a significant number of LMS cases may be “hiding” under the diagnosis of UPS.¹⁷ We recently described 3 novel molecular LMS subtypes that are distributed similarly over LMS of GYN and non-GYN origin. The Group 1 subtype shows an improved disease-specific survival compared to the other 2 groups that is independent of histologic grade¹. Group 1 comprises ~25% of all LMS and is defined by a shared pattern of gene expression, a distinct pattern of genomic changes, and reactivity for at least 3 of 5 immunohistochemistry (IHC) markers (ACTG2, CASQ2, CFL2, MYLK, SLMAP) as tested on 271 cases of LMS in TMAs. These IHC markers have not been well characterized in non-LMS sarcomas. Here we provide a characterization of these 5 markers across normal tissues, an additional 59 cases of LMS, and a wide range of 565 non-LMS soft tissue tumors from 44 diagnostic categories with a focus on UPS.

When analyzed individually, the 5 markers were found to be expressed in many sarcomas other than LMS. However, when analyzed by the same criteria used for the recognition of Group 1 LMS where a case is scored positive when at least 3 of 5 markers reacted, coordinate expression was seen in significant numbers of cases from only three diagnostic groups that included 22% of leiomyomas (n=22), 16% of gastrointestinal stromal tumors (n=43), and 18% of endometrial stromal sarcoma (n=11). In addition, 5% (n=57) of UPS showed a staining pattern similar to that seen in Group 1 LMS.

To further examine the possibility that Group 1 LMS constitutes a small part of cases diagnosed as UPS, we examined the expression of the top 500 genes from the Group 1 LMS expression

signature in 29 UPS by cDNA microarray. Unsupervised hierarchical clustering of the 29 UPS expression revealed that 2 (7%) had coordinated high levels of expression of genes from the Group 1 LMS signature, a rate similar to that seen by IHC analysis.

These findings show that Group 1 LMS IHC markers ACTG2, CASQ2, CFL2, MYLK, and SLMAP when coordinately expressed have specificity for a subset of LMS when compared to other sarcomas and may be useful for the recognition of Group 1 LMS cases within cases diagnosed as UPS.

Introduction

Leiomyosarcomas (LMS) represent the most common soft tissue sarcoma subtype, accounting for ~24% of all mesenchymal malignancies.²² At present subjective morphologic criteria, supported by immunostaining characteristics for actin and desmin, are used to define smooth muscle differentiation in sarcomas¹⁶. Morphologic and existing immunohistochemical tools have failed to define clinically relevant LMS subset criteria and currently they offer no opportunity to direct therapeutic options for these tumors. In addition, the most commonly used grading scheme from the FNCLCC (Federation Nationale des Centres de Lutte Contre le Cancer) has relatively weak prognostic power in extra-uterine LMS.^{5,7,11,12,18}

Recently, we used gene expression profiling, array-based comparative genomic hybridization (aCGH), and tissue microarray immunohistochemistry (TMA IHC) to define 3 novel molecular subtypes of LMS. One of these types, the Group 1 or “muscle-enriched” subtype, showed improved disease-specific survival when compared to the other 2 groups that was independent of histologic grade.¹ These Group 1 tumors express a large number of genes at high levels, compared to the other 2 groups, including several known oncogenes and genes involved in muscle differentiation. Identifying Group 1 LMS is thus of considerable prognostic and potentially therapeutic interest. Diagnosis of this subtype can be performed by demonstrating Group 1 tumors’ immunoreactivity for a novel set of biomarkers (ACTG2, CASQ2, CFL2, MYLK, SLMAP) selected on the basis of gene expression patterns. All five of these genes play a role in muscle contractile function and are involved to varying extents not only in smooth muscle differentiation, but also in cardiac and skeletal muscle development. When coordinately expressed, ACTG2, CASQ2, CFL2, MYLK, and SLMAP identify Group 1 LMS, but the reactivity of these markers has not yet been examined in other sarcomas.

Here we report the first large scale characterization of these 5 LMS Group 1 markers across a wide range of benign soft tissue tumors and sarcomas with a focus on UPS. The immunohistochemical staining results for 57 UPS were compared with previously unpublished gene expression profiles of 29 UPS to confirm the immunohistochemical analyses.

Methods

IHC staining with ACTG2 (1:2000, Novus Biologicals (Littleton, CO), cat# H00000072-A01), CASQ2 (1:100, GeneTex Inc (Irvine, CA) cat# GTX90833), CFL2 (1:100, Genetic Inc (Irvine, CA), cat# GTX92818), MYLK (1:25, GeneTex Inc (Irvine, CA) cat# GTX91044), and SLMAP (1:2500, GeneTex Inc (Irvine, CA) cat# GTX94163) was performed on tissue arrays TA-167 and TA-170 with 565 cores including sarcomas and benign soft tissue lesions from 44 diagnostic entities and normal tissues. Immunoreactivity was visualized using Vector's Vectastain Elite ABC kit (mouse) after citrate pretreatment for each antibody. The five antibodies were chosen because they detect gene products that were

among those defining the Group 1 subtype by gene expression profiling and because commercially available antibodies already existed¹. The cases on the tissue microarrays were derived from the Stanford University Department of Surgical Pathology's internal files and all diagnoses were rendered according to routine practices by department faculty. According to department practice, a diagnosis of UPS requires an absence of discernable lineage commitment (including smooth muscle differentiation) by morphology or immunohistochemistry. All five stains were additionally applied to an LMS tissue microarray, TA-201, containing 377 LMS. We have previously reported the immunohistochemical findings for TA-201¹; these stains were reviewed and verified for this study.

Staining was scored as positive (tumor cell cytoplasmic staining visible by low power), weak/absent, or uninterpretable. Only cores for which more than 3 of the 5 antibody stains were interpretable were included in analysis, and only those with ≥ 3 positive markers were considered to show a significant correlation with Group 1 LMS. Digital images from all TMA cores stained by immunohistochemistry used in this study are publically accessible on the website: <http://tma.stanford.edu/>.

For gene expression profiling studies, the expression of the top 500 genes from the Group 1 LMS expression signature¹ was evaluated in 29 cases of UPS on the same 44,000 spot cDNA microarray platform used to define the group 1 LMS cases. The 29 UPS cases used for gene expression analysis were centrally reviewed (CF). The cases were clustered together with the 51 LMS cases used in our previous study using expression for these 500 genes by hierarchical clustering.

Results

Staining in normal tissues

The Group 1 LMS gene expression profile was characterized by an abundance of genes known to be involved in muscle differentiation. As expected the antibodies selected from these gene lists showed reactivity with smooth muscle cells in a variety of sites but the reactive patterns were not uniform and showed significant variability depending on the site of origin of the smooth muscle samples (Figure 1).

ACTG2, CFL2, MYLK, SLMAP diffusely stained smooth muscle cell cytoplasm in the gastrointestinal tract, while ACTG2, CFL2, and SLMAP were positive in myometrium. Only ACTG2 and SLMAP were positive in smooth muscle from the bladder wall. ACTG2 and SLMAP were strongly positive in arterial and venous blood vessels from a variety of sites while CASQ2 showed variable reactivity with vessel walls.

Group 1 markers also stained other muscle subtypes. Cardiac muscle was positive for SLMAP, CFL2, ACTG2, and CASQ2. Interestingly, ACTG2 and CASQ2 demonstrated a more focal, punctate pattern of cytoplasmic reactivity in cardiac muscle when compared to the diffuse cytoplasmic reactivity of these markers in other positive tissues as well as the SLMAP and CFL2 staining pattern in cardiac muscle. Skeletal muscle reacted strongly with ACTG2, CASQ2, and SLMAP but showed variable staining with MYLK.

No staining for any of the 5 markers was appreciated in hematolymphoid tissue from the tonsil or in a variety of epithelial tissues including lung, thyroid, salivary gland, bowel mucosa, and kidney.

Staining in LMS

As reported previously, IHC staining revealed immunopositivity for at least 3 of the 5 Group 1 markers in 25% (84/330) of LMS (Figure 2). A much larger proportion of LMS tumors showed sporadic staining with one or two of the 5 markers (63%, 209/330). Of note, the proportion of tumors expressing at least 3 Group 1 markers was comparable for LMS of GYN (26%, 27/105) and non-GYN origin (25%, 57/225). Group 1 marker staining patterns were previously described for 271 LMS cases¹ and staining for an additional 59 cases is included here to reach a total of 330 LMS cases. The rationale for choosing a cut-off of at least three markers was derived primarily from gene expression profiling data, which identified the Group 1 tumor subset based on coordinate expression of 360 genes. While many tumors expressed individual Group 1 genes by immunohistochemistry, the concomitant expression of all five genes appeared to be relatively specific to a subset of leiomyosarcoma.¹ Because data for all five stains was not uniformly available for all cases due to technical difficulties (e.g. loss of cores, uninterpretable staining), a minimum requirement of 3 positive stains was chosen to maximize available data while still excluding cases of spurious staining. Using these criteria, the immunohistochemical studies identified a subset of LMS cases that represented a similar percentage of cases as identified through gene expression profiling.

Staining in other soft tissue tumor types

Group 1 marker expression was evaluated in a variety of other sarcomas and benign soft tissue processes (Table 1). The majority of lesions did not show coordinate expression of at least 3 markers and only 3 diagnostic groups showed staining in significant numbers of cases. These included 22% (5/22) of benign leiomyomas occurring in GYN (n=2/19) and non-GYN sites (n=3/3). Additionally, 16% of gastrointestinal stromal tumor (n=7/43) and 18% of endometrial stromal sarcomas (n=2/11) reacted with at least three Group 1 markers. The other sarcomas and benign mesenchymal processes failed to demonstrate positive staining with 3 Group 1 markers (see Table 1). Although negative for the Group 1 markers as a panel, in some instances non-LMS sarcoma subtypes expressed a single Group 1 LMS marker. Notably, single-marker expression was present in 38% of liposarcoma (n=26) and 13% of rhabdomyosarcoma (n=15). In these instances the identity of the positive marker varied across tumor types however trends emerged within certain tumor subtypes. For instance, 27% (7/26) of liposarcomas were positive for CFL2 but all were negative for SLMAP.

Staining and Gene Array Data in UPS

Expression of 3 Group 1 markers was seen in 5% of UPS (n=3/57). These cases were reviewed and were confirmed to be negative for the smooth muscle markers desmin (3/3) and smooth muscle actin (2/3). A much larger proportion (37%, 21/57) of cases showed positivity for at least 1 Group 1 marker, similar to the relatively high incidence of staining with a single marker seen in other non-LMS sarcomas. Even in UPS cases where all 5 markers were positive, staining intensity was diminished when compared to the most convincingly stained LMS cases and normal tissues (Figure 3). Additionally, although widespread marker positivity was not detected in a variety of other non-LMS sarcomas (with the exception of ESS and GIST, for which there is some biologic basis for smooth muscle differentiation and marker reactivity), the study included more UPS than any other individual sarcoma subtype, therefore cases of incidental or spurious reactivity would more likely occur in this category.

To further address the reactivity of LMS Group 1 in a subset of UPS gene expression profiling (GEP) was performed. Hierarchical clustering and principal component analysis were performed on a set of 29 UPS and 51 previously characterized Group 1 and non-Group

1 LMS¹ using the 500 genes that were used to identify Group 1 LMS in our previous study. The average expression of 500 Group 1 genes was calculated, and cases were then ranked according to this value. A cut-point was determined according to the average gene expression value at which the first non-Group 1 LMS case appeared on the rank list. Using this stringent cut-point, only rare cases (7%, 2/29) of UPS met criteria for positive Group 1 gene expression. If a much more permissive cut-point was used (e.g. the average gene expression value of the last Group 1 LMS case), 55% (16/29) of UPS qualified for inclusion.

Discussion

Leiomyosarcoma is a malignancy with an aggressive course and limited treatment options. At present, prognosis of LMS is predicted using traditional clinicopathologic features¹⁵ and the current chemotherapeutic armamentarium for these tumors, when uncontrolled, is based predominantly on doxorubicin and related therapies and results in only marginal improvements in survival. Although molecular biomarkers do not yet inform prognostication and treatment of LMS in the clinical setting, gene expression microarrays have already been employed to detect signatures of probable metastasis in these tumors.^{10,18} Additionally, macrophage infiltration¹⁸ and the CSF1 response signature⁷ have been identified by our group as poor prognostic factors in LMS. An improved understanding of the pathogenesis of LMS molecular subtypes could help further inform prognosis and shed light on chemotherapeutic vulnerabilities of LMS. Given the improved prognosis of Group 1 LMS when compared to other LMS subtypes, reliable distinction of Group 1 from both other LMS subtypes and other soft tissue sarcomas is critical. Our studies here are intended to determine whether 5 IHC markers shown previously to be useful for the distinction of Type I LMS (with good prognosis) could be useful in a clinical setting.

Our findings suggest that the novel markers ACTG2, CASQ2, CFL2, MYLK, and SLMAP are valuable tools in the diagnosis of Group 1 LMS, a subset of LMS cases that we previously defined by gene expression profiling.¹ The presence of strong Group 1 marker expression in 25% of LMS in the current study coincides with previously reported finding that Group 1 comprises ~25% of LMS.¹ When used in concert, these markers were also useful at distinguishing Group 1 LMS from a variety of other sarcomas. Individually, however, the antibody stains had variable reactivity profiles and not infrequently stained other non-LMS sarcomas. These findings suggest that while a panel of stains is a powerful tool for the diagnosis of Group 1 LMS, single markers are of only limited utility depending upon the differential diagnosis.

One frequently encountered diagnostic problem for which individual markers may be of value is in the distinction of LMS from de-differentiated liposarcoma. While diagnosis is straightforward in morphologically classic cases, differentiating LMS from liposarcoma may be difficult when the former entraps significant amounts of fat or the latter shows areas of divert differentiation. Expression SLMAP was highly specific (100%) for LMS when the differential was limited to LMS vs. liposarcoma. MYLK performed similarly, showing 96% specificity for LMS when applied in this focused differential.

The presence of strong Group 1 marker expression in significant numbers of ESS and GIST is intriguing. Smooth muscle differentiation is well-documented in both tumors. In the case of ESS, admixed areas of benign smooth muscle are relatively common and are not thought to influence prognosis so long as cytologic atypia is absent; the presence of any significant atypia in areas of smooth muscle differentiation excludes the diagnosis of ESS and places the lesion under consideration for a diagnosis of LMS.²⁰ Group 1 marker staining in bland smooth muscle components of ESS is not surprising, given that a subset of benign smooth

muscle tumors react with these immunostains, and should not represent a source of additional diagnostic difficulty in the distinction of ESS and LMS.

Group 1 marker staining was also noted in some GISTs. A subset of these GISTs has been shown to demonstrate histologic features of smooth muscle differentiation with desmin and actin positivity. While initial work suggested distinct differences in gene copy number between LMS and GIST,¹⁹ recent molecular studies have demonstrated common regions of 13q and 11q imbalance in GIST and LMS.²³ Further study is warranted to evaluate the significance of the morphologic, immunohistochemical, and molecular overlap of some GISTs with LMS.

Although the Group 1 subtype includes a greater proportion of histologically well-differentiated LMS than do the other LMS clusters, the prognostic significance of Group 1 was independent of grade¹ and the absence of strong Group 1 staining in the majority of leiomyomas provides argument against the supposition that the Group 1 subtype constitutes a better-differentiated form of LMS. In addition, these clinically well-behaving tumors paradoxically demonstrate increased levels of genomic complexity, with shared affected genes, when compared to other LMS.¹ Prior work has also shown that the most frequently lost region in Group 1 LMS is 2.5 MB on 1p36.32, which includes the *PRDM16* gene. The loss of *PRDM16* promotes differentiation of brown fat precursors into skeletal muscle and results in elevated expression of genes involved in smooth, cardiac, and skeletal muscle (including ACTG2, MYLK, CFL2, SLMAP, and CASQ2).^{1,21} The heterogeneity of muscle genes expressed in Group 1 tumors may help to explain the frequent overlap of individual Group 1 immunostains with other sarcoma subtypes.

Despite the increased genetic complexity of Group 1 LMS, patients with these tumors experience a survival benefit that is independent of histologic grade¹ suggesting that factors beyond degree of differentiation influence prognosis. The finding that the Group 1 phenotype connotes improved prognosis irrespective of differentiation is of particular interest in cases of UPS showing strong Group 1 marker expression by immunohistochemistry, particularly given the finding that a comparable percentage of UPS show high coordinated Group 1 gene expression on hierarchical clustering of cDNA microarrays. These markers may define a small population of Group 1 LMS-derived UPS with improved prognosis and could suggest future therapeutic targets. This is especially relevant given that myogenic differentiation usually confers worse prognosis with increased risk of metastasis and decreased overall survival in UPS.^{6,8} Although the rarity of Group 1 marker-positive UPS argues against the possibility that large numbers of Group 1 LMS “hide” within the UPS category, reappraisal of UPS with Group 1 features may provide prognostically useful information.

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Figure 1. Group 1 marker expression in normal tissues. A) ACTG expression in myometrium B) CASQ2 expression in skeletal muscle C) CFL2 expression in cardiac muscle D) MYLK expression in skeletal muscle E) SLMAP in myometrium

Figure 2. Group 1 marker expression in LMS. Twenty-five percent of LMS showed strong reactivity with at least 3 of the 5 Group 1 markers. A) ACTG B) CASQ2 C) CFL2 D) MYLK E) SLMAP

Figure 3.

Group 1 marker expression in UPS. Rare UPS expressed Group 1 markers however staining intensity and extent was diminished when compared to Group 1 leiomyosarcoma and normal muscle tissues. A) ACTG B) CASQ2 C) CFL2 D) MYLK E) SLMAP

Table 1

Expression of Group I leiomyosarcoma markers in soft tissue neoplasms

	+	++	+++
Adenosarcoma (n=1)	100%	0%	0%
ALT (n=5)	20%	0%	0%
Angiomyolipoma (n=8)	75%	25%	0%
Angiosarcoma (n=8)	13%	0%	0%
Carcinosarcoma (n=5)	40%	20%	0%
Chondrosarcoma (n=14)	29%	0%	0%
Clear cell sarcoma (n=3)	33%	33%	0%
DFSP (n=9)	0%	0%	0%
DRSCT (n=5)	0%	0%	0%
Elastofibroma (n=1)	0%	0%	0%
Embryonal Sarcoma (n=2)	50%	0%	0%
Enchondroma (n=4)	0%	0%	0%
Epithelioid Sarcoma (n=2)	50%	0%	0%
ESS (n=11)	54%	27%	18%
Ewing's Sarcoma (n=8)	0%	0%	0%
Facitis (n=5)	40%	0%	0%
Fibroadenoma (n=9)	0%	0%	0%
Fibroma of oral cavity (n=2)	0%	0%	0%
Fibroma of ovary (n=9)	11%	11%	11%
Fibroma of tendon sheath (n=6)	0%	0%	0%
Fibromatosis (n=25)	12%	0%	0%
Fibrosarcoma (n=2)	0%	0%	0%
Fibrous dysplasia (n=9)	0%	0%	0%
Fibroblastoma (n=4)	0%	0%	0%
GIST (n=43)	40%	19%	16%
Glomus tumor (n=5)	60%	0%	0%
Granular cell tumor (n=4)	0%	0%	0%
Hemangiopericytoma (n=8)	34%	25%	0%
Inflammatory pseudotumor (n=3)	0%	0%	0%
Myxoma (n=7)	0%	0%	0%
Myxofibrosarcoma (n=9)	11%	0%	0%
Leiomyoma (n=22)	91%	73%	23%
Leiomyosarcoma (n=330)	63%	54%	25%
Liposarcoma (n=26)	38%	8%	0%
MPNST (n=7)	0%	0%	0%
Neurofibroma (n=12)	0%	0%	0%
Nodular Fascitis (n=4)	50%	0%	0%

	+	++	+++
Osteosarcoma (n=14)	0%	0%	0%
Phyllodes tumor (n=2)	0%	0%	0%
Rhabdomyosarcoma (n=15)	13%	7%	0%
Schwannoma (n=22)	0%	0%	0%
SFT (n=19)	0%	0%	0%
STUMP (n=2)	100%	100%	100%
Synovial sarcoma (n=20)	5%	5%	0%
Undifferentiated pleomorphic sarcoma (n=57)	37%	14%	5%

KEY:

“+”= 1 stain strongly positive

“++”= 2 stains strongly positive

“+++”= 3 of stains strongly positive