Original Article

Relationships Between Highly Recurrent Tumor Suppressor Alterations in 489 Leiomyosarcomas

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BACKGROUND: Leiomyosarcoma (LMS) is the most common soft tissue and uterine sarcoma, but no standard therapy is available for recurrent or metastatic LMS. TP53, p16/RB1, and PI3K/mTOR pathway dysregulations are recurrent events, and some LMS express estrogen receptor (ER) and/or progesterone receptor (PR). To characterize relationships between these pathway perturbations, the authors evaluated protein expression in soft tissue and uterine nonprimary leiomyosarcoma (np-LMS), including local recurrences and distant metastases. **METHODS:** TP53, RB1, p16, and PTEN expression aberrations were determined by immunohistochemistry (IHC) in tissue microarrays (TMAs) from 227 np-LMS and a comparison group of 262 primary leiomyosarcomas (p-LMS). Thirty-five of the np-LMS had a matched p-LMS specimen in the TMAs. Correlative studies included differentiation scoring, ER and PR IHC, and *CDKN2A*/p16 fluorescence in situ hybridization. **RESULTS:** Dysregulation of TP53, p16/RB1, and PTEN was demonstrated in 90%, 95%, and 41% of np-LMS, respectively. PTEN inactivation was more common in soft tissue np-LMS than uterine np-LMS (55% vs 31%; $P = .0005$). Moderate-strong ER expression was more common in uterine np-LMS than soft tissue np-LMS (50% vs 7%; $P < .0001$). Co-inactivation of TP53 and RB1 was found in 81% of np-LMS and was common in both soft tissue and uterine np-LMS (90% and 74%, respectively). RB1, p16, and PTEN aberrations were nearly always conserved in p-LMS and np-LMS from the same patients. **CONCLUSIONS:** These studies show that nearly all np-LMS have TP53 and/or RB1 aberrations. Therefore, therapies targeting cell cycle and DNA damage checkpoint vulnerabilities should be prioritized for evaluations in LMS. ***Cancer* 2021;127:2666-2673**. © *2021 American Cancer Society*.

KEYWORDS: biomarker, cell cycle, DNA damage repair, immunohistochemistry, leiomyosarcoma (LMS), soft tissue, uterine, uterus.

INTRODUCTION

Leiomyosarcoma (LMS) is the most common sarcoma and is characterized by evidence of smooth muscle differentiation.[1,2] LMS most frequently arises in the uterus, intra-abdominal soft tissues and large blood vessels (retroperitoneum/pelvis and mesentery), and extremities, although it also arises in other anatomic locations.[3-5] Approximately 50% of cases metastasize, especially to the lungs, bone, soft tissues, and liver, with overall 5-year survival rates of 60% for extremity LMS, 20% to 30% for retroperitoneal LMS, and 40% for uterine LMS.[6,7]

Anatomic site, depth, histologic grade, and subtype (epithelioid, myxoid, and pleomorphic) are prognostic factors in LMS.[3,8-10] Loss of myogenic differentiation correlates with a poorer prognosis,[8] as do macrophage infiltration and a CSF1-dependent macrophage expression signature.[9,11,12] Molecular profiles based on gene expression profiling and array comparative genomic hybridization have defined molecular subtypes of LMS and enabled the development and validation of immunohistochemistry (IHC) biomarkers of metastasis and survival.[9,10] However, despite these advances, there are no standard therapies for metastatic LMS. Targeted therapies, particularly pazopanib,[13] and conventional cytotoxic agents (including doxorubicin, ifosfamide, gemcitabine, docetaxel, and trabectedin) have modest clinical activity,[14-17]

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but more effective strategies are needed for patients with advanced LMS.

Mutations dysregulating the TP53, p16/RB1, and PI3K/mTOR pathways—typically through biallelic inactivation of key tumor suppressors—are recurrent events in LMS [18-20] and identify opportunities for targeted therapies. [21] In addition, approximately 20% to 60% of LMS (predominantly those arising in the uterus) express estrogen receptor (ER) and/or progesterone receptor (PR), and a subset of these LMS are responsive to hormonal manipulations such as aromatase inhibition. [22] However, the frequencies with which these aberrations occur—individually and in association with each other—are not well understood within different clinicopathologic LMS subsets. The lack of highly effective targeted monotherapies underscores the need to identify rational combination therapy strategies.

Here, we evaluated LMS from 489 patients to determine the frequency of tumor suppressor aberrations dysregulating TP53, RB1, p16, and PTEN and to determine the co-occurrence of these aberrations with one another and with ER and PR expression. The studies highlight biological rationales for therapeutic targeting and cotargeting in LMS.

MATERIALS AND METHODS

Patient Samples

Cases were retrieved retrospectively from surgical pathology files at MD Anderson Cancer Center and contributed by patient advocacy groups to Stanford University to create tissue microarrays (TMAs), as previously published, [7,8,23] including 2 cores per sample. Representative hematoxylin-eosin–stained slides underwent expert pathology review by 3 of the authors (A.J.L., M.V.D.R., and E.G.D.). These studies were performed on 258 LMS from MD Anderson Cancer Center (53%) and on 231 LMS from Stanford University (47%). Nonprimary leiomyosarcomas (np-LMS) were evaluated in 227 patients and included local recurrences (recurrent LMS) in 80 patients and distant metastases (metastatic LMS) in 147 patients. In 35 of these patients, the corresponding primary tumor was also evaluated. Primary leiomyosarcoma (p-LMS) only was evaluated in an additional 262 patients. Clinicopathologic features are provided in Table 1 and Supporting Table 1.

The primary sites of origin were soft tissue (n = 245; 50%) and the uterus (n = 244; 50%). The primary sites of origin for soft tissue LMS included the retroperitoneum/pelvis (44%), extremities (19%), vascular sites (14%), the trunk (4%), and miscellaneous sites (18%). Histologic differentiation scores were assessed as described previously[8] and were well differentiated (WD; n = 59; 26%), moderately differentiated (MD; n = 103; 45%), or poorly differentiated (PD; n = 65; 29%). These studies were performed with the approval of the institutional review boards of all institutions involved.

TABLE 1. Clinicopathologic Characteristics of 489 LMS

Characteristic	Value
Age, median (range), y	52 (22-91)
Sex: female/male, No. (%)	403 (82)/86 (18)
Primary site, No. (%)	
Soft tissue	245 (50)
Retroperitoneal/pelvic	108 (44)
Extremity	47 (19)
Vascular	35 (14)
Trunk	11 (4)
Other miscellaneous sites	44 (18)
Uterine	244 (50)
Specimen, No. (%)	
Nonprimary LMS	227 (46)
Local recurrence	80 (35)
Distant metastasis	147 (65)
Primary LMS	262 (54)

Abbreviation: LMS, leiomyosarcoma.

Immunohistochemistry

IHC was performed on 4-μm-thick, formalin-fixed, paraffin-embedded TMA sections after pressure cooker antigen retrieval (Target Retrieval Solution, pH 6.1; Dako, Carpinteria, California) with antibodies and staining conditions summarized in Supporting Table 2. Positive and negative control tissues (placenta, smooth muscle, leiomyomata, and non-LMS sarcomas) were included on all sections. Stained TMA slides were imported to the Stanford Tissue Microarray Database (https://tma.im/cgi-bin/home.pl) and scored on slide and on line by 4 of the authors (I.M.S., M.V.D.R., A.M.E., and J.A.F.). Cases without staining in LMS cells and internal controls (admixed blood vessels and inflammatory cells) were removed from the analysis, as were cases with uninterpretable or equivocal stains in LMS cells. Scoring categories are summarized by marker in Supporting Table 2.

TP53 expression was scored as negative (a complete absence of staining in tumor cells with a positive internal control), normal (weak positive nuclear staining in 10%-50% of tumor cells), or overexpressed (strong and diffuse nuclear staining in >50% of tumor cells; Fig. 1). RB1 expression was scored as negative (a complete absence of staining in tumor cells with a positive internal control) or normal (positive nuclear expression in >10%

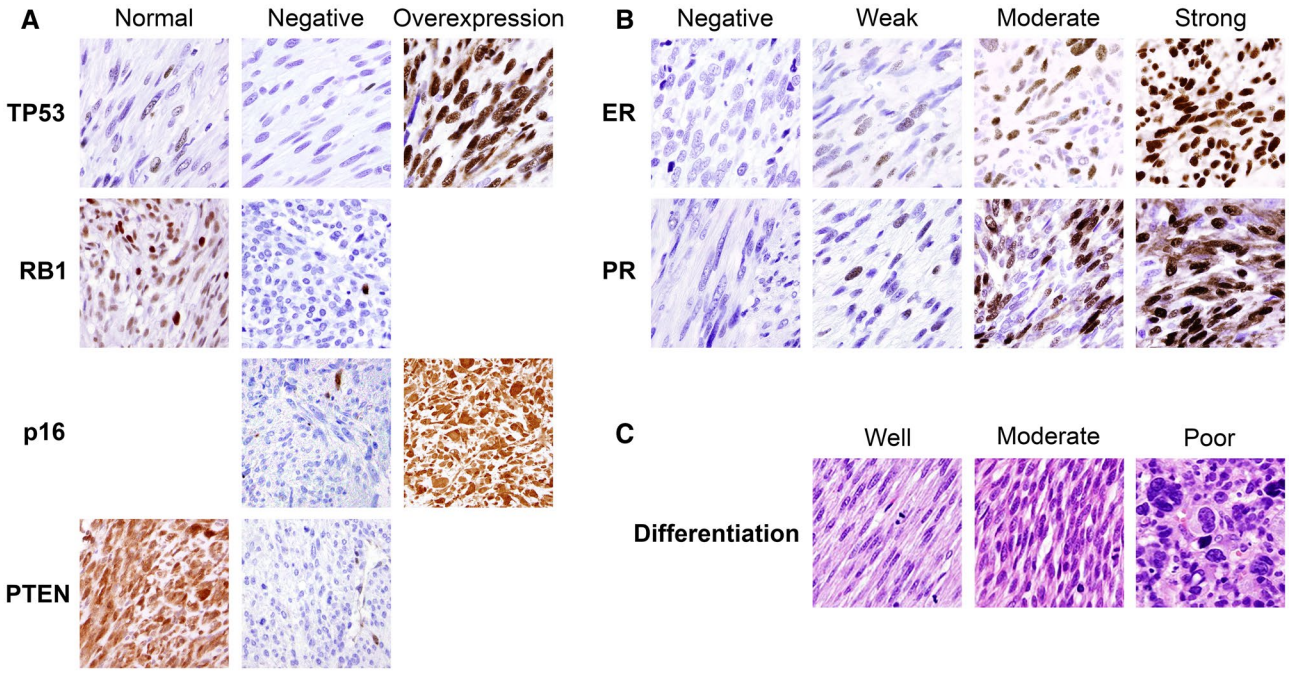

Figure 1. (A) Representative leiomyosarcomas illustrating immunohistochemical staining categories for TP53 (scored as normal, negative, or overexpressed), RB1 (normal or negative), p16 (negative or overexpressed), and PTEN (normal or negative). (B) Expression of ER and PR was scored as negative (a complete absence of staining), weak (weak staining in all cells or moderate to strong staining in <50% of cells), moderate (moderate to strong staining in 50%-89% of cells), or strong (moderate to strong staining in ≥90% of cells). (C) Representative leiomyosarcomas illustrating histologic tumor differentiation based on nuclear atypia and pleomorphism. ER indicates estrogen receptor; PR, progesterone receptor.

of tumor cells; Fig. 1). p16 expression was scored as negative (a complete absence of staining in tumor cells with a positive internal control) or overexpressed (diffuse cytoplasmic and nuclear staining in 80%-100% of tumor cells and with greater intensity in comparison with benign controls; Fig. 1). p16 diffuse overexpression is known to be a biomarker of RB1 inactivation and represents a feedback loop from the loss of RB1-mediated *CDKN2A*/p16 transcriptional repression.[24] Fewer than 5% of specimens had scattered (5%-10%) p16-positive cells, which were interpreted as equivocal and removed from further analysis because the implications of this staining pattern are unknown and the histologic criteria for normal p16 staining are not well defined. PTEN expression was scored as negative (a complete absence of staining in tumor cells with a positive internal control) or normal (positive nuclear and/or cytoplasmic staining in >10% of tumor cells; Fig. 1).

Expression of the hormone receptors ER and PR was scored as negative (a complete absence of staining in tumor cells), weakly positive (weak nuclear staining in all tumor cells or moderate to strong nuclear staining in <50% of tumor cells), moderately positive (moderate to strong nuclear staining in 50%-89% of tumor cells), or strongly positive (moderate to strong nuclear staining in ≥90% of tumor cells; Fig. 1).

Fluorescence In Situ Hybridization

Bacterial artificial chromosome (BAC) fluorescence in situ hybridization (FISH) probes were labeled by random octamer priming.[25] A 101,154nt BAC (RP11-149I2) for *CDKN2A* (p16 coding sequence) was labeled with biotin. BACs covering the 9q *MUSK* locus (RP11-476F1, RP11-665P9, RP11-115I1) were labeled with digoxigenin as a chromosome 9 reference probe. BACs were obtained from BAC/PAC Resources (Children's Hospital, Oakland, California). LMS cores were scored as having *CDKN2A*/p16 homozygous deletion when more than 40% of neoplastic cells had no *CDKN2A*/p16 target probe FISH signals but had 1 or more *MUSK* control probe signals.

Statistical Analysis

IHC protein expression data were analyzed with STATA/IC 15.1 software. The 2-sided Fisher exact test was used to compare frequencies between groups. An unadjusted P value $\leq$ .05 was considered statistically significant.

TABLE 2. TP53, RB1, p16, and PTEN Aberrations and Moderate-Strong Hormone Receptor Expression in Nonprimary LMS (n = 227) and Primary LMS (n = 262)

	All Nonprimary LMS, %	All Primary LMS, %	P^a	Nonprimary LMS, %			Primary LMS, %		
				Soft Tissue	Uterine	P^a	Soft Tissue	Uterine	P^a
Inactivation									
TP53	90	84	.07	92	88	.36	87	81	.3
RB1	92	86	.09	97	87	.03	90	82	.08
p16	11	19	.02	11	11	1.00	15	25	.05
p16 (with retained RB1)	2	7	.04	3	2	.66	4	10	.07
PTEN	41	44	.57	55	31	**.0005**	47	41	.36
Moderate-strong expression									
ER	31	23	.03	7	50	**<.0001**	9	39	**<.0001**
ER and PR	17	12	.19	4	26	**<.0001**	4	22	**<.0001**

Abbreviations: ER, estrogen receptor; LMS, leiomyosarcoma; PR, progesterone receptor.
[a]Two-sided Fisher exact test. The unadjusted significance level was $P \leq .05$; the Bonferroni-adjusted significance level was $P \leq .0071$ (highlighted in bold).

Significance levels were adjusted for multiple comparisons with the Bonferroni method, with *P* values of ≤.0071 (Table 2) and ≤.0056 (Table 3), respectively, considered statistically significant.

RESULTS

Aberrations in Metastatic LMS

The IHC staining patterns for this LMS series are shown in Figure 1. Results of IHC expression profiles for the 489 LMS (227 np-LMS and 262 p-LMS) are summarized in Tables 2 and 3. TP53 inactivation was identified in 90% of np-LMS, with comparable frequencies in soft tissue and uterine cases (92% vs 88%) and in WD, MD, and PD cases (91% vs 91% vs 88%; Table 2 and Supporting Table 3). A complete loss of TP53 expression was found in 57% of np-LMS, whereas TP53 overexpression—consistent with a *TP53* change-of-function mutation—was found in 33% of np-LMS. RB1 inactivation was demonstrated in 92% of np-LMS, including 97% and 87% of soft tissue and uterine cases, respectively, with comparable frequencies in WD, MD, and PD cases (96% vs 91% vs 89%; Table 2 and Supporting Table 3). p16 expression was aberrant in all interpretable np-LMS: 11% had a loss of p16 expression, and the remaining 89% had p16 overexpression (Table 2), with similar frequencies in soft tissue and uterine cases and in WD, MD, and PD cases (Supporting Table 3). p16 inactivation with retained RB1 was demonstrated in 2% of np-LMS, including 3% of soft tissue cases and 2% of uterine cases (Table 2). PTEN inactivation was demonstrated in 41% of np-LMS. PTEN inactivation was more common in soft tissue LMS than uterine np-LMS (55% vs 31%; $P = .0005$).

Moderate-strong expression of ER was observed in 31% of np-LMS, with higher frequencies in uterine np-LMS than soft tissue np-LMS (50% vs 7%; $P < .0001$; Table 2). Moderate-strong expression of ER and PR was identified in 17% of np-LMS and was more frequent in uterine np-LMS than soft tissue np-LMS (26% vs 4%; $P < .0001$; Table 2). The frequencies of TP53, RB1, p16, and PTEN aberrations in the entire series of 489 LMS (np-LMS and p-LMS) were similar in cases with moderate-strong ER in comparison with those with lower levels of ER expression. Namely, the frequencies of abnormalities for LMS with moderate-strong ER expression and LMS with negative-weak ER expression were 87% and 86%, respectively, for TP53; 82% and 91%, respectively, for RB1; 18% and 14%, respectively, for p16; and 33% and 47%, respectively, for PTEN (Supporting Table 1).

Pathway codysregulations are summarized in Table 3. Concurrent inactivation of TP53 and RB1 was demonstrated in 81% of np-LMS and was frequent in both soft tissue and uterine np-LMS (90% and 74%, respectively; Table 3).

Tumor suppressor expression alterations were equally frequent in soft tissue np-LMS from female and male patients (Supporting Table 4). Likewise, these alterations were comparably frequent in distant metastases (metastatic LMS) and local recurrences (recurrent LMS), with the exception of RB1 inactivation, which was more common in metastatic LMS ($P = .0044$; Supporting Table 5). Tumor suppressor alterations were also comparably frequent in soft tissue np-LMS originating at different anatomic sites (Supporting Table 6).

Pathway Aberrations in p-LMS

The frequencies of TP53, p16, RB1, and PTEN expression alterations in 262 p-LMS were comparable to those in the np-LMS in this series (Table 2). Moderate-strong

TABLE 3. Associations of TP53, RB1, p16, and PTEN Aberrations and Moderate-Strong ER Expression in Nonprimary LMS (n = 227)

Association[a]	All Nonprimary LMS, %	Nonprimary LMS, %		P[b]
		Soft Tissue	Uterine	
TP53 + RB1	81	90	74	.01
TP53 + p16	1	2	1	.42
TP53 + PTEN	38	54	26	**.0001**
TP53 + ER	29	8	45	**<.0001**
RB1 + PTEN	41	55	30	**.001**
RB1 + ER	26	7	42	**<.0001**
p16 + PTEN	1	1	0	.45
p16 + ER	1	1	0	.45
PTEN + ER	8	2	12	**.005**

Abbreviations: ER, estrogen receptor; LMS, leiomyosarcoma.
[a]ER, moderate-strong expression; p16, loss of expression with retained RB1 expression; PTEN, loss of expression; RB1, loss of expression; TP53, loss of expression or overexpression.
[b]Two-sided Fisher exact test. The unadjusted significance level was $P \leq .05$; the Bonferroni-adjusted significance level was $P \leq .0056$ (highlighted in bold).

ER expression was demonstrable in 23% of p-LMS and was just as common (31%) in np-LMS. However, moderate-strong ER expression was more common in uterine p-LMS than soft tissue p-LMS (39% vs 9%; $P < .0001$; Table 2).

Aberrations in Paired p-LMS and np-LMS

TP53, p16, RB1, and PTEN expression was evaluated in paired p-LMS and np-LMS from 35 patients (Supporting Table 7). These included 20 soft tissue LMS and 15 uterine LMS. Tumor suppressor alterations were highly concordant within each p-LMS/np-LMS pair. Namely, expression patterns for RB1, p16, and PTEN were concordant in 100%, 100%, and 97% of the p-LMS/np-LMS pairs, respectively. TP53 expression was concordant in 82% of the p-LMS/np-LMS pairs.

CDKN2A*/p16 FISH*

CDKN2A/p16 FISH was evaluated in 221 LMS, of which 19% had a loss of p16 expression and 81% had p16 overexpression. *CDKN2A*/p16 homozygous deletion was identified in 6% of these cases, each of which had a loss of p16 expression.

DISCUSSION

Even though it is the most common soft tissue and uterine sarcoma, there are no clinically accepted prognostic or predictive biomarkers for LMS, and targeted therapy approaches based on a biological rationale remain to be developed. Genomic aberrations of p16, RB1, TP53, and PTEN are recurrent features of LMS, but the demonstrated frequency of these aberrations varies considerably among reported studies. [18,19,21] We evaluated p16, RB1, TP53, and PTEN protein expression—together with ER and PR expression—in 489 soft tissue and uterine LMS, which included 227 cases with np-LMS (35 of which had a paired primary tumor) and a comparator set of 262 cases represented by p-LMS only.

The evaluations in this large LMS study set demonstrate dysregulation of TP53 and p16/RB1 in nearly all soft tissue and uterine np-LMS. Loss of PTEN expression was demonstrated in 41% of these np-LMS. Recent p-LMS genome and transcriptome evaluations by The Cancer Genome Atlas Research Network (80 LMS: soft tissue [n = 53] and uterine [n = 27]) demonstrated *TP53* deep deletions and mutations in 9% and 50% of cases, respectively; *RB1* deep deletions and mutations in 14% and 15% of cases, respectively; and *PTEN* deep deletions in 13% of cases. [19] Integrative LMS genome and transcriptome evaluations from Chudasama et al [18] (49 LMS: soft tissue [n = 39] and uterine) demonstrated *TP53* biallelic genomic inactivation in 92% of cases, *RB1* biallelic inactivation in 94%, *CDKN2A*/p16 inactivation in 8%, and *PTEN* inactivation in 57%. These frequencies are comparable to those for protein inactivation in our current study, where we demonstrate TP53 and RB1 inactivation in 90% and 92% of np-LMS, respectively (Table 2). *TP53* and *RB1* are inactivated by myriad genomic mechanisms, including single-nucleotide variants, indels, copy number alterations, and chromosomal rearrangements, and it is likely that these diverse genomic alterations are more systematically detected in some studies than others. [18] These challenges highlight the continued need for well-validated biomarkers at the protein level to complement and extend the insights from genomic assays. As 1 example cited previously, the systematic evaluation of *RB1* biallelic genomic inactivation is hampered by the large size of this gene and the extremely varied inactivation mechanisms. We show that the complementary approach of well-validated RB1 IHC demonstrates RB1 loss in 92% of LMS and is reassuringly confirmed, in most cases, by diffuse p16 overexpression resulting from RB1-mediated feedback loops (Fig. 1 and Supporting Table 1).

The aims of this study were to 1) determine frequencies of key tumor suppressor aberrations in a large study group of soft tissue and uterine np-LMS, 2) determine associations between protein alterations and thereby define frequencies of np-LMS with rationales for combination therapies, and 3) determine whether random samples (TMA cores) from p-LMS are useful indicators of alterations in concurrent or subsequent np-LMS. One motivation for this study was to determine the frequency

of np-LMS having a loss of p16 expression with retained RB1 expression, a profile providing a rationale for CDK4/6 inhibition. [21,26] Although loss of p16 and/or RB1 expression was found in 95% of np-LMS, only 2% of cases had a loss of p16 expression with retained RB1 expression. These findings show that alternative strategies for targeting dysregulated p16/RB1 are needed.

Of the more than 70 clinicopathologic types of soft tissue and uterine sarcoma, LMS is the only type occurring with substantial frequency in both the hereditary retinoblastoma and Li-Fraumeni syndromes. [27,28] Kleinerman et al reported that 14 of 16 sarcomas outside radiation fields in survivors of hereditary retinoblastoma were LMS, [29] with uterine LMS particularly common in this setting. [30] These observations demonstrate that RB1 and TP53 inactivation, beyond being essential tumorigenic events in most LMS, are predisposing and initiating events in an LMS subset. Likewise, the finding of RB1 and TP53 inactivation in 86% and 84% of TMA cores, respectively, from p-LMS (Table 2) provides further evidence that these are early events in LMS tumorigenesis. In contrast, LMS has not been reported in association with germline *PTEN* mutations (eg, in patients with Cowden syndrome) [31] or germline *CDKN2A*/p16 mutations (eg, kindreds with a familial predisposition to melanoma or pancreatic cancer). [32] In sum, LMS is unusual among soft tissue and uterine sarcomas in having both RB1 and TP53 inactivation as initiating or early events in tumorigenesis. These observations suggest that dysregulation of cell cycle and DNA damage response (DDR) checkpoints is key to LMS initiation and early progression.

It is striking that RB1 dysregulation and TP53 dysregulation are coupled essential events in LMS genesis and progression, as demonstrated by co-inactivation of RB1 and TP53 in 90% and 74% of soft tissue and uterine np-LMS, respectively (Table 3). RB1 and TP53 have overlapping biological functions, particularly in cell cycle regulation, and recent studies show that co-inactivation of these essential checkpoint proteins can be crucial in tumorigenesis. Flesken-Nikitin et al [33] showed that concurrent RB1 and TP53 inactivation fostered ovarian cancer in mouse models, and Zhou et al [34] showed the same in the development of prostate cancer. By contrast, Zhou et al showed that inactivation of either RB1 or TP53 fostered only premalignant epithelial proliferation in the prostate. Extending this observation to clinical samples, Nyquist et al [35] demonstrated that combined RB1 and TP53 inactivation in prostate cancer was associated with increased proliferation, increased DDR, and reduced survival. Notably, Nyquist et al also demonstrated that prostate cancer models with concurrent RB1/TP53-inactivation, although highly resistant to conventional therapies, acquired vulnerabilities associated with elevated replication stress, which resulted in sensitivity to DDR inhibition. These studies highlight therapeutic strategies that target combined RB1/TP53 inactivation by increasing genotoxicity to untenable levels and thereby converting RB1/TP53 inactivation into a liability. [35] The same concepts are likely relevant in LMS, which is characterized by chromosomal instability (CIN) and complexity. [19,36] Recent studies have demonstrated that CIN in many LMS results from homologous recombination (HR) defects, engendering "BRCAness" vulnerabilities that provide rationales for therapeutic strategies involving PARP inhibition. [18] Notably, HR and nonhomologous end joining (NHEJ) are the major complementary pathways responsible for repairing double-strand DNA damage, and TP53 is a regulator of NHEJ, which, therefore, assists in constraining CIN to levels that are not inordinately genotoxic for the cancer cells. The aforementioned observations suggest that the near-universal RB1/TP53 inactivation in LMS, coupled with HR defects, creates opportunities for therapeutically maximizing replication stress, for example, by combination therapies involving DNA-damaging agents and DDR inhibitors. Other rational strategies might include combinations of DDR inhibitors, such as combinations of PARP and NHEJ pathway inhibitors, thereby maximizing HR dysregulation while antagonizing compensatory NHEJ responses, which are also partially compromised because of TP53 inactivation.

Although CDK4/6 inhibition appears relevant in only a minority of np-LMS, approaches that provide synthetic lethal interactions with RB1 deficiency have been described recently in carcinoma models. Zhao et al [37] showed that pharmacologic inhibition of the E3-ligase subunit SKP2 (which is repressed by RB1) blocks growth of RB1-deficient prostate or small cell lung cancers. Other groups have demonstrated that RB1-deficient cancers are hyperdependent on Aurora kinase A (AURKA) and Aurora kinase B (AURKB); this creates vulnerabilities to AURKA and AURKB inhibitors, as has been demonstrated in uterine LMS models. [23,38,39] These strategies merit further validation in LMS preclinical models and warrant evaluation in clinical trials because most np-LMS have an intrinsic RB1 deficiency. As shown in Table 3, synthetic lethal interactions with RB1 deficiency warrant particular scrutiny as therapeutic strategies in combination with DDR inhibitors in TP53/RB1-deficient LMS (81% of np-LMS), with mTOR inhibitors in RB1/PTEN-deficient LMS (41%

of np-LMS), and with ER pathway inhibitors in LMS with RB1 deficiency and moderate-strong ER expression (42% of uterine np-LMS).

As stated previously, we had expected that LMS with p16 inactivation and retained RB1 expression, representing candidates for therapeutic CDK4/6 inhibition, would be more numerous among np-LMS than proved to be the case. p16 inactivation with retained RB1 expression was found in 2% of np-LMS and in 7% of p-LMS (Table 2). We performed *CDKN2A*/p16 FISH to validate the IHC findings, particularly to determine whether IHC failed to demonstrate p16 inactivation in a subset of cases with *CDKN2A*/p16 homozygous deletion. The FISH correlates showed that all 6% of LMS with demonstrable *CDKN2A*/p16 homozygous deletion were p16-negative by IHC, and they thereby supported the IHC findings. Furthermore, most RB1-deficient LMS overexpressed p16 diffusely and strongly (Fig. 1 and Supporting Table 2); this represents a known feedback mechanism in cancers with *RB1* genomic inactivation and retained *CDKN2A*/p16. [24]

Comparing soft tissue np-LMS and uterine np-LMS, our evaluations revealed comparable rates of RB1 inactivation (97% and 87%) and statistically significant differences in inactivation of PTEN (55% and 31%; Table 2). As expected, uterine np-LMS expressed ER and PR more often than soft tissue np-LMS (Table 2). Nonetheless, moderate-strong ER expression was demonstrated in 7% of soft tissue np-LMS, all of which were retroperitoneal/pelvic or abdominal/chest wall in origin (Supporting Tables 1 and 4).

Comparisons of TP53, RB1, p16, and PTEN expression in p-LMS and np-LMS from 35 patients showed that the expression patterns for RB1 and p16 in every patient and for PTEN in all but 1 patient were conserved between the p-LMS and np-LMS pairs (Supporting Table 7). TP53 expression patterns were concordant in 82% of the p-LMS/np-LMS pairs, and 4 of the 6 TP53 expression discrepancies involved classifications of "normal" versus "loss"; this is a challenging IHC distinction because normal TP53 expression is a weakly positive IHC stain, which is often only subtly different from TP53 loss (Fig. 1). These findings are consistent with TP53, RB1, p16, and PTEN aberrations as early and nonevolving events in most LMS and show that the use of these proteins as predictive biomarkers for np-LMS can generally be based on an analysis of the corresponding p-LMS.

In summary, the protein expression profiles herein are the largest series reported for LMS, and they are in keeping with recent integrative genomic and transcriptomic analyses showing that TP53 inactivation and RB1 inactivation are near-universal aberrations in both soft tissue and uterine LMS. [18] These studies showed PTEN deficiency in approximately half of np-LMS, but more commonly in soft tissue np-LMS than uterine np-LMS, whereas moderate-strong ER expression was found in half of uterine np-LMS and in fewer than 10% of soft tissue np-LMS. p16 deficiency with retained RB1 expression—a pattern providing a rationale for CDK4/6 inhibition—was found in only 2% of np-LMS. Little expression heterogeneity was detected between primary and metastatic disease from the same patients, and this was in keeping with the early and even initiating roles of these protein dysregulations in LMS genesis.

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CONFLICT OF INTEREST DISCLOSURES

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AUTHOR CONTRIBUTIONS

Inga-Marie Schaefer: Study concept and design; acquisition, analysis, and interpretation of data; writing–original draft; and writing–review and editing. **Meijun Z. Lundberg:** Acquisition, analysis, and interpretation of data and writing–review and editing. **Elizabeth G. Demicco:** Study concept and design; administrative, technical, or material support; acquisition of data; and writing–review and editing. **Joanna Przybyl:** Acquisition, analysis, and interpretation of data and writing–review and editing. **Magdalena Matusiak:** Acquisition, analysis, and interpretation of data and writing–review and editing. **Frédéric Chibon:** Study concept and design and writing–review and editing. **Davis R. Ingram:** Acquisition, analysis, and interpretation of data and writing–review and editing. **Jason L. Hornick:** Administrative, technical, or material support; development of methodology; and writing–review and editing. **Wei-Lien Wang:** Acquisition, analysis, and interpretation of data and writing–review and editing. **Sebastian Bauer:** Study concept and design and writing–review and editing. **Laurence H. Baker:** Study concept and design; acquisition, analysis, and interpretation of data; and writing–review and editing. **Alexander J. Lazar:** Study concept and design; administrative, technical, or material support; acquisition, analysis, and

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