DR. INGA-MARIE SCHAEFER (Orcid ID : 0000-0001-9710-5500)

Author Manuscript

Article type : Original Article

Relationships Between Highly Recurrent Tumor Suppressor Alterations in 489 Leiomyosarcomas

Running Title: Tumor Suppressor Alterations in 489 LMS

Inga-Marie Schaefer, MD[1], Meijun Z. Lundberg, MD[1]*, Elizabeth G. Demicco, MD, PhD[2]*, Joanna Przybyl, PhD[3], Magdalena Matusiak, PhD[3], Frédéric Chibon, PhD[4], Davis R. Ingram[5], BS, Jason L. Hornick, MD, PhD[1], Wei-Lien Wang, MD[5], Sebastian Bauer, MD[6], Laurence H. Baker, DO[7], Alexander J. Lazar, MD, PhD[5], Matt van de Rijn, MD, PhD[3], Adrian Mariño-Enríquez, MD, PhD[1], and Jonathan A. Fletcher, MD[1]

*Contributed equally

[1]Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA

[2]Department of Pathology and Laboratory Medicine, Sinai Health System & Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada

[3]Department of Pathology, Stanford University School of Medicine, Stanford, California, USA

[4]INSERM U1037, Cancer Research Center of Toulouse, Department of Pathology, Institut Claudius Régaud, IUCT-Oncopole, Toulouse, France

[5]Departments of Pathology and Translational Molecular Pathology, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/CNCR.33542

This article is protected by copyright. All rights reserved

[6]Department of Medical Oncology, Sarcoma Center, West German Cancer Center, University Duisburg-Essen, Medical School, Essen, Germany; DKTK Partner Site Essen and German Cancer Consortium (DKTK), Heidelberg, Germany

[7]Department of Internal Medicine, University of Michigan, Ann Arbor, Michigan, USA

Correspondence

Jonathan A. Fletcher, MD, Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, 20 Shattuck Street, Boston, MA, USA; E-mail: jfletcher@bwh.harvard.edu; Phone: +1 (617) 732-7883

Funding Support

This research was supported by the SARC (Sarcoma Alliance for Research through Collaboration) LMSARC research fund (J. A. Fletcher, L. H. Baker, M. van de Rijn, and A. Marino-Enriquez), the Liddy Shriver Sarcoma Initiative (J. A. Fletcher, S. Bauer, F. Chibon, M. van de Rijn), the National Institutes of Health/National Cancer Institute K08 CA241085 grant (I.-M. Schaefer), the Leiomyosarcoma Support & Direct Research Foundation (M. van de Rijn) and the National LeioMyoSarcoma Foundation (M. van de Rijn).

Conflict of Interest Disclosures

S. Bauer has received honoraria from Novartis, Pfizer, Bayer, PharmaMar, and GlaxoSmithKline; served in advisory or consultancy role for Blueprint Medicines, Bayer, Lilly, Deciphera, Exelixis, Janssen-Cilag, Plexxikon, and Nanobiotix; received institutional research funding from Blueprint Medicines, Novartis, and Incyte; and received travel and accommodation funding from PharmaMar. The remaining authors declare no conflict of interest.

Authors' Contributions

- Inga-Marie Schaefer: Study concept and design; acquisition, analysis and interpretation of data; writing-original draft; writing-review and editing.
- Meijun Z. Lundberg: Acquisition, analysis and interpretation of data; writing-review and editing.

This article is protected by copyright. All rights reserved

Author Manuscript

- Elizabeth G. Demicco: Study concept and design; administrative, technical, or material support; acquisition of data; writing-review and editing.
- Joanna Przybyl: Acquisition, analysis and interpretation of data; writing-review and editing.
- Magdalena Matusiak: Acquisition, analysis and interpretation of data; writing-review and editing.
- Frédéric Chibon: Study concept and design; writing-review and editing.
- Davis R. Ingram: Acquisition, analysis and interpretation of data; writing-review and editing.
- Jason L. Hornick: Administrative, technical, or material support; development of methodology writing-review and editing.
- Wei-Lien Wang: Acquisition, analysis and interpretation of data; writing-review and editing.
- Sebastian Bauer: Study concept and design; writing-review and editing.
- Laurence H. Baker: Study concept and design; acquisition, analysis and interpretation of data; writing-review and editing.
- Alexander J. Lazar: Study concept and design; administrative, technical, or material support, acquisition, analysis and interpretation of data; writing-review and editing.
- Matt van de Rijn: Study concept and design; development of methodology; administrative, technical, or material support, acquisition, analysis and interpretation of data; writing-review and editing.
- Adrian Mariño-Enríquez: Study concept and design; acquisition, analysis and interpretation of data; writing-review and editing.
- Jonathan A. Fletcher: Study concept and design; acquisition, analysis and interpretation of data; writing-original draft; writing-review and editing.

Acknowledgements

The authors would like to thank Mei Zheng, Immunohistochemistry Lab at the Department of Pathology, Brigham and Women's Hospital, Boston, MA, for immunohistochemical staining.

Precis

Aberrations of TP53, p16/RB1, and PTEN are found, respectively, in 90%, 95%, and 41% of metastatic soft tissue and uterine leiomyosarcomas, with PTEN inactivation being more common

This article is protected by copyright. All rights reserved

Author Manuscript

in soft tissue cases. These key aberrations are generally conserved between primary and metastatic disease in a given patient.

Total number of: Text pages: 25; Tables: 3; Figures: 1; Supporting files for publication: 8 (7 Supporting Tables)

ABSTRACT

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 and/or progesterone receptors (ER, PR). To characterize relationships between these pathway perturbations, we evaluated protein expression in soft tissue and uterine non-primary LMS (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 LMS (p-LMS). 35 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, respectively, in 90%, 95%, and 41% of np-LMS. PTEN inactivation was more common in soft tissue than uterine np-LMS (55% vs. 31%, P=0.0005). Moderate-strong ER expression was more common in uterine than soft tissue np-LMS (50% vs. 7%, P<0.0001). 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. **Conclusion:** 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.

Keywords:

Leiomyosarcoma, LMS, soft tissue, uterus, uterine, biomarker, immunohistochemistry, cell cycle, DNA damage repair.

This article is protected by copyright. All rights reserved

Author Manuscript

INTRODUCTION

Leiomyosarcoma (LMS) is the most common sarcoma and is characterized by evidence of smooth muscle differentiation.[1, 2] LMS most frequently arise in the uterus, intra-abdominal soft tissues and large blood vessels (retroperitoneum/pelvis, and mesentery) and extremities, although LMS also arise 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, 20-30% 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 poorer prognosis,[8] as does 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 development and validation of immunohistochemical (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] 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 20-60% of LMS (predominantly those arising in the uterus), express estrogen and/or progesterone receptors (ER, 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 co-occurrence of these aberrations with each other and with ER and PR expression. The studies highlight biologic rationales for therapeutic targeting and co-targeting in LMS.

This article is protected by copyright. All rights reserved

Author Manuscript

MATERIALS AND METHODS

Patient samples

Cases were retrieved retrospectively from surgical pathology files at MD Anderson Cancer Center (MDACC) and contributed from patient advocacy groups to Stanford University, to create tissue microarrays (TMAs) as previously published,[7, 8, 23] including two cores per sample. Representative hematoxylin and eosin (H&E)-stained slides underwent expert pathology review (A. J. Lazar, M. van de Rijn, E. G. Demicco). These studies were performed on 258 LMS from MDACC (53%) and 231 LMS from Stanford University (47%). Non-primary LMS (np-LMS) were evaluated in 227 patients, including local recurrences (r-LMS) in 80 patients and distant metastases (m-LMS) in 147 patients. In 35 of these patients, the corresponding primary tumor was also evaluated. Primary LMS (p-LMS) only was evaluated in an additional 262 patients. Clinicopathologic features are provided in Table 1 and Supporting Table 1.

Primary sites of origin were soft tissue (N=245; 50%) and uterus (N=244; 50%). Primary sites of origin for soft tissue LMS included retroperitoneum/pelvis (44%), extremities (19%), vascular (14%), 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 Board of all institutions involved.

Immunohistochemistry

IHC was performed on 4-µm-thick formalin-fixed paraffin-embedded TMA sections following pressure cooker antigen retrieval (Target Retrieval Solution; pH 6.1; Dako, Carpinteria, CA, USA) 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 I.-M. Schaefer, M. van de Rijn, A. Mariño-Enríquez, and J. A. Fletcher. Cases without staining in LMS cells and internal controls (admixed blood vessels and inflammatory cells) were removed from analysis, as were cases with uninterpretable or equivocal stains in the LMS cells. Scoring categories are summarized by marker in Supporting Table 2.

This article is protected by copyright. All rights reserved

Author Manuscript

TP53 expression was scored as negative (complete absence of staining in tumor cells with positive internal control) vs. 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 (complete absence of staining in tumor cells with positive internal control) vs. normal (positive nuclear expression in >10% of tumor cells) (Fig. 1). p16 expression was scored as negative (complete absence of staining in tumor cells, with positive internal control) vs. overexpressed (diffuse cytoplasmic and nuclear staining in 80-100% of tumor cells and with greater intensity than in benign controls) (Fig. 1). p16 diffuse overexpression is known to be a biomarker of RB1 inactivation, representing a feedback loop from 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, given that 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 (complete absence of staining in tumor cells with positive internal control) vs. 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 (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 (FISH)

BAC 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, CA). 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 one or more *MUSK* control probe signals.

Author Manuscript

This article is protected by copyright. All rights reserved

Statistical Analysis

IHC protein expression data were analyzed using STATA/IC 15.1 software. The two-sided Fisher's exact test was used to compare frequencies between groups. An unadjusted *P* value of ≤0.05 was considered statistically significant. Significance levels were adjusted for multiple comparisons using the Bonferroni method, with *P* values of ≤0.0071 (Table 2) and ≤0.0056 (Table 3), respectively, considered statistically significant.

RESULTS

Aberrations in metastatic leiomyosarcoma

The IHC staining patterns for this LMS series are shown in Fig. 1. Results of IHC expression profiles in 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). Complete loss of TP53 expression was found in 57% of np-LMS, whereas TP53 overexpression – consistent with *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, and with comparable frequency 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 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 and 2% of uterine cases (Table 2). PTEN inactivation was demonstrated in 41% of np-LMS. PTEN inactivation was more common in soft tissue than in uterine np-LMS (55% vs. 31%; *P*=0.0005).

Moderate-strong expression of ER was observed in 31% of np-LMS, with higher frequencies in uterine than soft tissue np-LMS (50% vs. 7%; *P*<0.0001) (Table 2). Moderate-strong expression of ER and PR was identified in 17% of np-LMS and was more frequent in uterine than soft tissue np-LMS (26% vs. 4%; *P*<0.0001) (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 compared to those with lower levels of ER expression. Namely, frequencies of abnormalities for ER moderate-strong vs. ER negative-weak LMS were 87% vs.

This article is protected by copyright. All rights reserved

Author Manuscript

86% for TP53; 82% vs. 91% for RB1; 18% vs. 14% for p16; and 33% vs. 47% for PTEN (Supporting Table 1).

Pathway co-dysregulations 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%) (Table 3).

Tumor suppressor expression alterations were equally frequent in soft tissue np-LMS from female vs. male patients (Supporting Table 4). Likewise, these alterations were comparably frequent in distant metastases (m-LMS) vs. local recurrences (r-LMS), with the exception of RB1 inactivation which was more common in m-LMS (P=0.0044; Supporting Table 5). Tumor suppressor alterations were also comparably frequent in soft tissue np-LMS originating in different anatomic sites (Supporting Table 6).

Pathway aberrations in primary leiomyosarcoma

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 ER expression was demonstrable in 23% of primary LMS and was just as common (31%) in non-primary LMS. However, moderate-strong ER expression was more common in uterine than soft tissue p-LMS (39% vs. 9%; P <0.0001) (Table 2).

Aberrations in paired primary and non-primary leiomyosarcoma

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 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, respectively, in 100%, 100%, and 97% of the p-LMS/np-LMS pairs. TP53 expression was concordant in 82% of the p-LMS/np-LMS pairs.

***CDKN2A*/p16 fluorescence *in-situ* hybridization (FISH)**

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

This article is protected by copyright. All rights reserved

Author Manuscript

DISCUSSION

Despite being 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 biologic 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 that 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 primary LMS genome and transcriptome evaluations by The Cancer Genome Atlas (TCGA) Research Network (80 LMS: soft tissue LMS, N=53; uterine LMS, N=27) demonstrated *TP53* deep deletions and mutations, respectively, in 9% and 50% of cases, *RB1* deep deletions and mutations in 14% and 15% of cases, and *PTEN* deep deletions in 13% of cases.[19] Integrative LMS genome and transcriptome evaluations from Chudasama et al. (49 LMS: soft tissue LMS, N=39; uterine LMS) demonstrated *TP53* biallelic genomic inactivation in 92% of cases, *RB1* biallelic inactivation in 94%, *CDKN2A*/p16 inactivation in 8%, and *PTEN* inactivation in 57%.[18] These frequencies are comparable to those for protein inactivation in our present study, where we demonstrate TP53 and RB1 inactivation, respectively, in 90% and 92% of np-LMS (Table 2). *TP53* and *RB1* are inactivated by myriad genomic mechanisms, including single nucleotide variants, indels, copy number alterations (CNAs), and chromosomal rearrangements, and it is likely 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 one example given above, 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 (Figure 1, Supporting Table 1).

This article is protected by copyright. All rights reserved

Author Manuscript

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, thereby defining 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 loss of p16 expression and retained RB1 expression, a profile providing 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 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 clinicopathological 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 a subset of LMS. Likewise, the finding of RB1 and TP53 inactivation, respectively, in 86% and 84% of TMA cores from p-LMS (Table 2) provides further evidence that these are early events in LMS tumorigenesis. In contrast, LMS have not been reported in association with germline *PTEN* mutations, e.g., in patients with Cowden syndrome,[31] or germline *CDKN2A*/p16 mutations, e.g., kindreds with 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 checkpoints is key to LMS initiation and early progression.

It is striking that RB1 and TP53 dysregulation are coupled essential events in LMS genesis and progression, as demonstrated by co-inactivation of RB1 and TP53 in 90% vs. 74% of soft tissue vs. uterine np-LMS, respectively (Table 3). RB1 and TP53 have overlapping biologic 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. showed that concurrent RB1 and TP53 inactivation fostered ovarian cancer in mouse models[33] and Zhou

This article is protected by copyright. All rights reserved

Author Manuscript

et al. showed the same in development of prostate cancer.[34] By contrast, Zhou et al. showed that inactivation of either RB1 or TP53 fostered only premalignant epithelial proliferation in the prostrate.[34] Extending this observation to clinical samples, Nyquist et al. demonstrated that combined RB1 and TP53 inactivation in prostate cancer was associated with increased proliferation, increased DNA damage response, and reduced survival.[35] 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, resulting in sensitivity to DNA damage response inhibition.[35] These studies highlight therapeutic strategies that target combined RB1/TP53 inactivation by increasing genotoxicity to untenable levels, 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 demonstrate 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 non-homologous 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 aren't inordinately genotoxic for the cancer cells. The abovementioned observations suggest that the near-universal RB1/TP53 inactivation in LMS, coupled with HR defects, create 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 due to 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. 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.[37] Other groups demonstrated that RB1-deficient cancers are hyperdependent on Aurora kinases A and B (AURKA and AURKB), creating vulnerabilities to AURKA and AURKB inhibitors, as have been demonstrated in uterine LMS models.[23, 38, 39] These strategies merit further validations in LMS preclinical models and warrant evaluation in clinical trials, given that

This article is protected by copyright. All rights reserved

Author Manuscript

most np-LMS have 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); mTOR inhibitors in RB1/PTEN-deficient LMS (41% of np-LMS); and ER pathway inhibitors in LMS with RB1 deficiency and moderate-strong ER expression (42% of uterine np-LMS).

As stated above, 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 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, thereby supporting the IHC findings. Furthermore, most RB1-deficient LMS overexpressed p16 diffusely and strongly (Figure 1 and Supporting Table 2), representing a known feedback mechanism in cancers with *RB1* genomic inactivation and retained *CDKN2A*/p16.[24]

Comparing soft tissue and uterine np-LMS, our evaluations revealed comparable rates of RB1 inactivation (97% and 87%) and statistically significant differences in inactivation of PTEN (55% vs. 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 vs. np-LMS from 35 patients showed that the expression patterns for RB1 and p16 in every patient, and for PTEN in all but one 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 four of the six TP53 expression discrepancies involved classifications of “normal” vs. “loss”, which is a challenging IHC distinction, given that normal TP53 expression is a weakly-positive IHC stain that is often only subtly different from TP53 loss (Fig. 1). These findings are consistent with TP53, RB1, p16 and PTEN aberrations as early and non-evolving events in most LMS and show

This article is protected by copyright. All rights reserved

Author Manuscript

that use of these proteins as predictive biomarkers for np-LMS can generally be based on analysis of the corresponding p-LMS.

In sum, 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 and RB1 inactivation are near-universal aberrations in both soft tissue and uterine LMS.[18] These studies show PTEN deficiency in approximately half of np-LMS, but more commonly in soft tissue than uterine np-LMS, whereas moderate-strong ER expression is 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 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, in keeping with the early and even initiating roles of these protein dysregulations in LMS genesis.

References

1. Toro JR, Travis LB, Wu HJ, Zhu K, Fletcher CD, Devesa SS. Incidence patterns of soft tissue sarcomas, regardless of primary site, in the surveillance, epidemiology and end results program, 1978-2001: An analysis of 26,758 cases. *Int J Cancer*. 2006;119(12):2922-2930.
2. Mastrangelo G, Coindre JM, Ducimetiere F, et al. Incidence of soft tissue sarcoma and beyond: a population-based prospective study in 3 European regions. *Cancer*. 2012;118(21):5339-5348.
3. WHO Classification of Tumours Editorial Board. *Soft Tissue and Bone Tumours*. 5th ed, Volume 3, WHO Classification of Tumours. World Health Organization; 2020.
4. WHO Classification of Tumours Editorial Board. *Tumours of Female Reproductive Organs*. 4th ed, Volume 6, WHO Classification of Tumours. World Health Organization; 2014.
5. Seagle BL, Sobecki-Rausch J, Strohl AE, Shilpi A, Grace A, Shahabi S. Prognosis and treatment of uterine leiomyosarcoma: A National Cancer Database study. *Gynecol Oncol*. 2017;145(1):61-70.
6. Schaefer IM, Fletcher CD. Diagnostically Challenging Spindle Cell Neoplasms of the Retroperitoneum. *Surg Pathol Clin*. 2015;8(3):353-374.

This article is protected by copyright. All rights reserved

Author Manuscript

7. Lusby K, Savannah KB, Demicco EG, et al. Uterine leiomyosarcoma management, outcome, and associated molecular biomarkers: a single institution's experience. *Ann Surg Oncol*. 2013;20(7):2364-2372.

8. Demicco EG, Boland GM, Brewer Savannah KJ, et al. Progressive loss of myogenic differentiation in leiomyosarcoma has prognostic value. *Histopathology*. 2015;66(5):627-638.

9. Guo X, Jo VY, Mills AM, et al. Clinically Relevant Molecular Subtypes in Leiomyosarcoma. *Clin Cancer Res*. 2015;21(15):3501-3511.

10. 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(5):1190-1196.

11. Lee CH, Espinosa I, Vrijaldenhoven S, et al. Prognostic significance of macrophage infiltration in leiomyosarcomas. *Clin Cancer Res*. 2008;14(5):1423-1430.

12. Espinosa I, Beck AH, Lee CH, et al. Coordinate expression of colony-stimulating factor-1 and colony-stimulating factor-1-related proteins is associated with poor prognosis in gynecological and nongynecological leiomyosarcoma. *Am J Pathol*. 2009;174(6):2347-2356.

13. van der Graaf WT, Blay JY, Chawla SP, et al. Pazopanib monotherapy in the treatment of pretreated,for metastatic uterinesoft-tissue sarcoma: (PALETTE): a single-center retrospective study. J Gynecol Oncol. 2018;29: e3randomised, double-blind, placebo-controlled phase 3 trial. *Lancet*. 2012;379(9829):1879-1886.

14. Collins IM, Thomas DM. Novel approaches to treatment of leiomyosarcomas. *Curr Oncol Rep*. 2011;13(4):316-322.

15. Van Glabbeke M, van Oosterom AT, Oosterhuis JW, et al. Prognostic factors for the outcome of chemotherapy in advanced soft tissue sarcoma: an analysis of 2,185 patients treated with anthracycline-containing first-line regimens--a European Organization for Research and Treatment of Cancer Soft Tissue and Bone Sarcoma Group Study. *J Clin Oncol*. 1999;17(1):150-157.

16. Karavasilis V, Seddon BM, Ashley S, Al-Muderis O, Fisher C, Judson I. Significant clinical benefit of first-line palliative chemotherapy in advanced soft-tissue sarcoma: retrospective

This article is protected by copyright. All rights reserved

Author Manuscript

analysis and identification of prognostic factors in 488 patients. *Cancer*. 2008;112(7):1585-1591.

17. Demetri GD, Chawla SP, von Mehren M, et al. Efficacy and safety of trabectedin in patients with advanced or metastatic liposarcoma or leiomyosarcoma after failure of prior anthracyclines and ifosfamide: results of a randomized phase II study of two different schedules. *J Clin Oncol*. 2009;27(25):4188-4196.
18. Chudasama P, Mughal SS, Sanders MA, et al. Integrative genomic and transcriptomic analysis of leiomyosarcoma. *Nat Commun*. 2018;9(1):144.
19. Cancer Genome Atlas Research Network. Comprehensive and Integrated Genomic Characterization of Adult Soft Tissue Sarcomas. *Cell*. 2017;171(4):950-965 e28.
20. Perot G, Chibon F, Montero A, et al. Constant p53 pathway inactivation in a large series of soft tissue sarcomas with complex genetics. *Am J Pathol*. 2010;177(4):2080-2090.
21. Elvin JA, Gay LM, Ort R, et al. Clinical Benefit in Response to Palbociclib Treatment in Refractory Uterine Leiomyosarcomas with a Common *CDKN2A* Alteration. *Oncologist*. 2017;22(4):416-421.
22. Leitao MM, Jr., Hensley ML, Barakat RR, et al. Immunohistochemical expression of estrogen and progesterone receptors and outcomes in patients with newly diagnosed uterine leiomyosarcoma. *Gynecol Oncol*. 2012;124(3):558-562.
23. Brewer Savannah KJ, Demicco EG, Lusby K, et al. Dual targeting of mTOR and aurora-A kinase for the treatment of uterine Leiomyosarcoma. *Clin Cancer Res*. 2012;18(17):4633-4645.
24. Romagosa C, Simonetti S, Lopez-Vicente L, et al. p16(Ink4a) overexpression in cancer: a tumor suppressor gene associated with senescence and high-grade tumors. *Oncogene*. 2011;30(18):2087-2097.
25. Lux ML, Rubin BP, Biase TL, et al. KIT extracellular and kinase domain mutations in gastrointestinal stromal tumors. *Am J Pathol*. 2000;156(3):791-795.

Author Manuscript

This article is protected by copyright. All rights reserved

26. Bohm MJ, Marienfeld R, Jager D, et al. Analysis of the CDK4/6 Cell Cycle Pathway in Leiomyosarcomas as a Potential Target for Inhibition by Palbociclib. *Sarcoma*. 2019;2019:3914232.

27. Ognjanovic S, Olivier M, Bergemann TL, Hainaut P. Sarcomas in *TP53* germline mutation carriers: a review of the IARC TP53 database. *Cancer*. 2012;118(5):1387-1396.

28. Venkatraman L, Goepel JR, Steele K, Dobbs SP, Lyness RW, McCluggage WG. Soft tissue, pelvic, and urinary bladder leiomyosarcoma as second neoplasm following hereditary retinoblastoma. *J Clin Pathol*. 2003;56(3):233-236.

29. Kleinerman RA, Tucker MA, Abramson DH, Seddon JM, Tarone RE, Fraumeni JF, Jr. Risk of soft tissue sarcomas by individual subtype in survivors of hereditary retinoblastoma. *J Natl Cancer Inst*. 2007;99(1):24-31.

30. Francis JH, Kleinerman RA, Seddon JM, Abramson DH. Increased risk of secondary uterine leiomyosarcoma in hereditary retinoblastoma. *Gynecol Oncol*. 2012;124(2):254-259.

31. Tan MH, Mester JL, Ngeow J, Rybicki LA, Orloff MS, Eng C. Lifetime cancer risks in individuals with germline PTEN mutations. *Clin Cancer Res*. 2012;18(2):400-407.

32. Goldstein AM, Fraser MC, Struewing JP, et al. Increased risk of pancreatic cancer in melanoma-prone kindreds with $p16^{INK4}$ mutations. *N Engl J Med*. 1995;333(15):970-974.

33. Flesken-Nikitin A, Choi KC, Eng JP, Shmidt EN, Nikitin AY. Induction of carcinogenesis by concurrent inactivation of p53 and Rb1 in the mouse ovarian surface epithelium. *Cancer Res*. 2003;63(13):3459-3463.

34. Zhou Z, Flesken-Nikitin A, Corney DC, et al. Synergy of p53 and Rb deficiency in a conditional mouse model for metastatic prostate cancer. *Cancer Res*. 2006;66(16):7889-7898.

35. Nyquist MD, Corella A, Coleman I, et al. Combined *TP53* and *RB1* Loss Promotes Prostate Cancer Resistance to a Spectrum of Therapeutics and Confers Vulnerability to Replication Stress. *Cell Rep*. 2020;31(8):107669.

This article is protected by copyright. All rights reserved

Author Manuscript

36. Fletcher JA, Morton CC, Pavelka K, Lage JM. Chromosome aberrations in uterine smooth muscle tumors: potential diagnostic relevance of cytogenetic instability. *Cancer Res*. 1990;50(13):4092-4097.

37. Zhao H, Iqbal NJ, Sukrithan V, et al. Targeted Inhibition of the E3 Ligase $SCF^{Skp2/Cks1}$ Has Antitumor Activity in *RB1*-Deficient Human and Mouse Small-Cell Lung Cancer. *Cancer Res*. 2020;80(11):2355-2367.

38. Oser MG, Fonseca R, Chakraborty AA, et al. Cells Lacking the *RB1* Tumor Suppressor Gene Are Hyperdependent on Aurora B Kinase for Survival. *Cancer Discov*. 2019;9(2):230-247.

39. Gong X, Du J, Parsons SH, et al. Aurora A Kinase Inhibition Is Synthetic Lethal with Loss of the *RB1* Tumor Suppressor Gene. *Cancer Discov*. 2019;9(2):248-263.

FIGURES

Figure 1.

Representative leiomyosarcomas (LMS) 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) (A). Expression of estrogen receptor (ER) and progesterone receptor (PR) was scored as negative (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) (B). Representative LMS illustrating histologic tumor differentiation based on nuclear atypia and pleomorphism (C).

Author Manuscript

This article is protected by copyright. All rights reserved

Table 1.

Clinicopathologic characteristics of 489 LMS.

Characteristic		
Age	Median years (range)	52 (22-91)
Sex	Female/Male	403/86 (82%/18%)
Primary site	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	Non-primary LMS	227 (46%)
	Local recurrence	80 (35%)
	Distant metastasis	147 (65%)

Author Manuscript

This article is protected by copyright. All rights reserved

Table 2.

TP53, RB1, p16, and PTEN aberrations and moderate-strong hormone receptor expression in non-primary (N=227) vs. primary (N=262) LMS.

	All non-primary LMS	All primary LMS	P*	Non-primary LMS		P*	Primary LMS		P*
				Soft tissue	Uterine		Soft tissue	Uterine	
Inactivation									
TP53	90%	84%	0.07	92%	88%	0.36	87%	81%	0.3
RB1	92%	86%	0.09	97%	87%	0.03	90%	82%	0.08
p16	11%	19%	0.02	11%	11%	1.00	15%	25%	0.05
p16 (w/ retained RB1)	2%	7%	0.04	3%	2%	0.66	4%	10%	0.07
PTEN	41%	44%	0.57	55%	31%	**0.0005**	47%	41%	0.36
Moderate-strong expression									
ER	31%	23%	0.03	7%	50%	**<0.0001**	9%	39%	**<0.0001**
ER and PR	17%	12%	0.19	4%	26%	**<0.0001**	4%	22%	**<0.0001**

*Two-sided Fisher's exact test; unadjusted significance level: P≤0.05; Bonferroni-adjusted significance level: P≤0.0071 (highlighted in bold)

Author Manuscript

This article is protected by copyright. All rights reserved

Table 3.

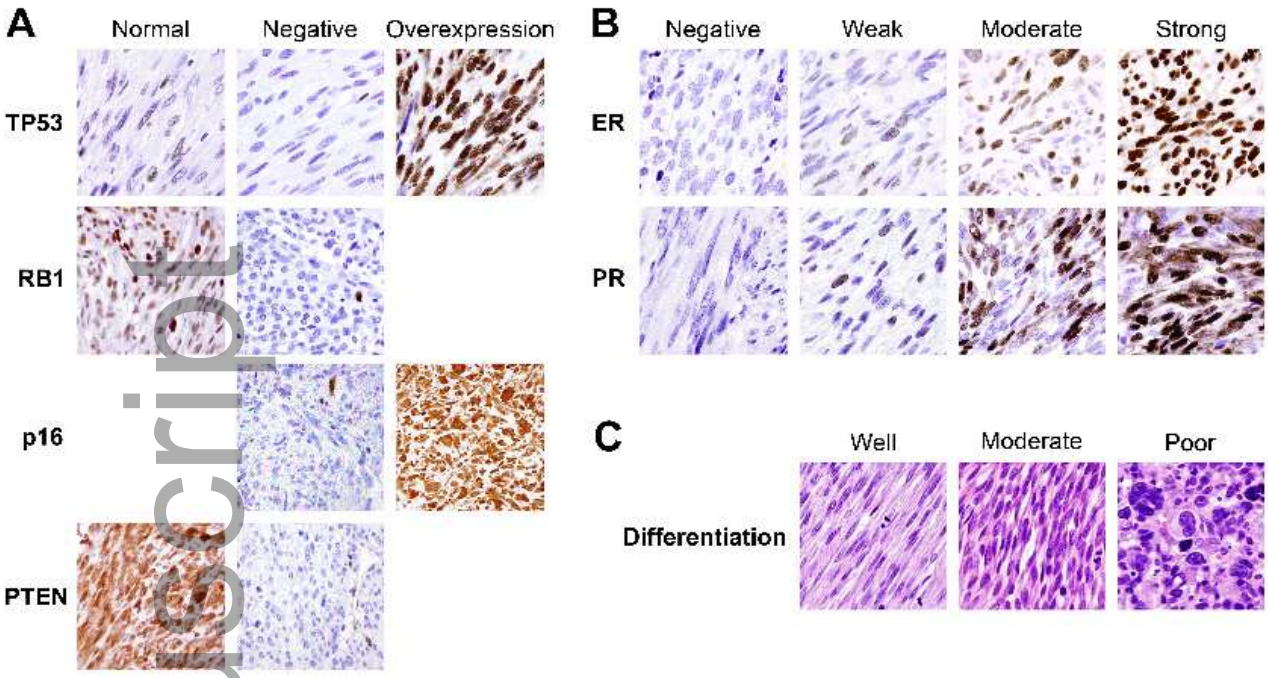
Associations of TP53, RB1, p16, and PTEN aberrations and moderate-strong ER expression in non-primary LMS (N=227).

Association	All non-primary LMS	Non-primary LMS		P*
		Soft tissue	Uterine	
TP53 + RB1	81%	90%	74%	0.01
TP53 + p16	1%	2%	1%	0.42
TP53 + PTEN	38%	54%	26%	**0.0001**
TP53 + ER	29%	8%	45%	**<0.0001**
RB1 + PTEN	41%	55%	30%	**0.001**
RB1 + ER	26%	7%	42%	**<0.0001**
p16 + PTEN	1%	1%	0%	0.45
p16 + ER	1%	1%	0%	0.45
PTEN + ER	8%	2%	12%	**0.005**

TP53: loss of expression or overexpressed; RB1: loss of expression; p16: loss of expression with retained RB1 expression; PTEN: loss of expression; ER: moderate-strong expression; *Two-sided Fisher's exact test; unadjusted significance level: P≤0.05; Bonferroni-adjusted significance level: P≤0.0056 (highlighted in bold)

Author Manuscript

This article is protected by copyright. All rights reserved



cncr_33542_f1.tif

Author Manuscript

This article is protected by copyright. All rights reserved