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HPV51-associated Leiomyosarcoma

A Novel Class of TP53/RB1-Wildtype Tumor With Predilection for the Female Lower Reproductive Tract

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Abstract: Inactivating mutations in tumor suppressor genes *TP53* and *RB1* are considered central drivers in leiomyosarcomas (LMSs). In high-risk human papillomavirus (HPV)-related tumors, a similar functional outcome is achieved through oncoproteins E6 and E7, which inactivate the p53 and RB1 proteins, respectively. Here, we hypothesized that HPV infection could provide an alternative mechanism for tumorigenesis in a subset of *TP53*/*RB1*-wildtype LMS. We evaluated tumor samples from 2585 consecutive unique patients carrying a diagnosis of gynecologic or soft tissue LMS. Tumor DNA and available RNA were analyzed by hybrid-capture-based next-generation sequencing/comprehensive genomic profiling of 406 genes and transcripts (FoundationOneHeme). Of the initial 2585 cases, we excluded 16 based on the presence of molecular alterations that are considered defining for sarcomas other than LMS. In the remaining 2569 cases, we searched for LMS that were *TP53*/*RB1*-wildtype (n = 486 of 2569; 18.9%). We also searched LMS tumors for HPV sequences that we then classified into genotypes by de novo assembly of nonhuman sequencing reads followed by alignment to the RefSeq database. Among *TP53*/*RB1*-wildtype LMS, we identified 18 unique cases harboring HPV sequences. Surprisingly, most (n = 11) were HPV51-positive, and these 11 represented all HPV51-positive tumors in our entire LMS database (n = 11 of 2569; 0.4%). The absence of genomic alterations in *TP53* or *RB1* in HPV51-positive LMS represented a marked difference from HPV51-negative LMS (n = 2558; 0% vs. 72% [$P < 0.00001$], 0% vs. 53% [$P = 0.0002$]). In addition, compared with HPV51-negative LMS, HPV51-positive LMS were significantly enriched for genomic alterations in *ATRX* (55% vs. 24%, $P = 0.027$) and *TSC1* (18% vs. 0.6%, $P = 0.0047$). All HPV51-positive LMS were in women; median age was 54 years at surgery (range: 23 to 74 y). All known primary sites were from the gynecologic tract or adjacent anogenital area, including 5 cases of vaginal primary site. Histology was heterogeneous, with evaluable cases showing predominant epithelioid (n = 5) and spindle (n = 5) morphology. In situ hybridization confirmed the presence of high-risk HPV E6/E7 mRNA in tumor cells in three of three evaluable cases harboring HPV51 genomic sequences. Overall, in our pan-LMS analysis, HPV reads were identified in a subset of *TP53*/*RB1*-wildtype LMS. For all HPV51-associated LMS, the striking absence of any detectable *TP53* or *RB1* mutations and predilection for the female lower reproductive tract supports our hypothesis that high-risk HPV can be an alternative tumorigenic mechanism in this distinct class of LMS.

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Reviewed and approved by Western IRB (Protocol No. 20152817) and University of California San Francisco Institutional Review Board (Protocol No. 11-05569).

Conflicts of Interest and Source of Funding: E.A.W., M.M., J.Y.T., M.C.H., D.A.M., B.B.S., B.M.A., J.S.R., S.H.R., D.I.L., and J.A.E. are employees or consultants of Foundation Medicine Inc., a wholly owned subsidiary of Roche Holdings Inc. and Roche Finance Ltd, and these employees have equity interest in an affiliate of these Roche entities. For the remaining authors none were declared.

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Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's website, www.ajsp.com.

Key Words: leiomyosarcoma, vagina, comprehensive genomic profiling, HPV, *ATRX*

(*Am J Surg Pathol* 2022;46:729–741)

Leiomyosarcoma (LMS), a malignant neoplasm defined by smooth muscle differentiation, is the most common gynecologic (GYN) sarcoma and can also arise in soft tissues elsewhere.[1,2] GYN LMS arises most frequently in the uterine wall and only rarely as a primary vaginal neoplasm.[3–5] GYN LMS is aggressive and resists standard therapy, with high rates of recurrence and progression; five-year survival for women with metastatic disease at presentation is only 10% to 15%.[6,7] Stage of disease, as defined by the International Federation of Gynecology and Obstetrics (FIGO)[8] or the American Joint Committee on Cancer,[9] is an important prognostic factor at the time of diagnosis.[1] Surgery is the standard of care for localized tumors, with hormonal and cytotoxic chemotherapy reserved for advanced stages.[10] Inactivating mutations in tumor suppressor genes *TP53* and *RB1* are considered central drivers in the majority of LMS.[11]

In high-risk (hr) human papillomavirus (HPV)-related tumors, a similar functional outcome is achieved through HPV oncoproteins E6 and E7, which inactivate the protein products p53 and RB1, respectively.[12] Genotypes of hrHPV, such as HPV16 and HPV18, are associated with the development of a subset of carcinomas, including cervical, anogenital, and a proportion of oropharyngeal carcinomas.[12–14] A potential role for hrHPV in the development of non-epithelial tumors, such as LMS, has not been defined.

Here, we hypothesized that HPV infection could provide an alternative mechanism for tumorigenesis in a distinct subset of *TP53*/*RB1*-wildtype LMS. To evaluate our hypothesis, we surveyed a large database of tumors previously diagnosed as LMS for recurrent HPV genome reads that occur in the absence of detectable mutations in *TP53* or *RB1*. Strikingly, we discovered a novel recurrent HPV51-positive genomic signature that was restricted to LMSs from lower female genital tract that were both *TP53*- and *RB1*-wildtype.

METHODS

Clinical Cohort and Comprehensive Genomic Profiling

Comprehensive genomic profiling was performed in a Clinical Laboratory Improvement Amendments (CLIA)-certified, College of American Pathologists (CAP)-accredited laboratory (Foundation Medicine Inc., Cambridge, MA). Approval for this study, including a waiver of informed consent and a Health Insurance Portability and Accountability Act (HIPAA) waiver of individual authorization, was obtained from the Western Institutional Review Board (Protocol No. 20152817). Our cohort initially included all GYN and soft tissue LMS referred to Foundation Medicine for comprehensive genomic profiling as part of clinical care (n = 2585 unique cases, including 12 of confirmed vaginal primary site). Board-certified pathologists on staff at Foundation Medicine reviewed the submitted pathologic diagnosis and examined routine hematoxylin and eosin (H&E)-stained slides of each case before sequencing. Molecular results were also reviewed by on-staff pathologists, and 16 cases were excluded from the final LMS cohort owing to the presence of molecular alterations that are considered defining for sarcomas other than LMS, meaning *BCOR-ZC3H7B* (n = 6), *PDGFB-COL1A1* (n = 3), *STAT6-NAB2* (n = 2), *GREB1-SS18* (n = 1), *SS18-SSX1* (n = 1), *SS18-SSX2* (n = 1), and *EWSR1-PATZ1* (n = 1) rearrangements, and *BCOR* internal tandem duplication (n = 1), for a final cohort of 2,569 LMS.

Before molecular analyses, sections were macrodissected to achieve > 20% estimated percent tumor nuclei in each case, where percent tumor nuclei is defined as 100 times the number of tumor cells divided by total number of nucleated cells.

For genomic assessments, ≥ 60 ng DNAs was extracted from 40 μm sections cut from formalin-fixed, paraffin-embedded tissue blocks. The samples were assayed by adaptor ligation hybrid capture, performed for all coding exons of 236 (v1), 315 (v2), or 405 (v3) cancer-related genes plus select introns from 19 (v1), 28 (v2), or 31 (v3) genes frequently rearranged in cancer (Supplemental Table 1, Supplemental Digital Content 1, http://links.lww.com/PAS/B306).[15,16] For samples with available RNA (n = 2,038), targeted RNA-seq was performed for analysis of rearrangements in 265 genes.[16] Sequencing of captured libraries was performed using the Illumina sequencing system to a mean exon coverage depth of targeted regions of > ×500, and sequences were analyzed for genomic alterations (GAs), including short variant alterations (base substitutions, insertions, and deletions), copy number alterations (focal amplifications and homozygous deletions), and select gene fusions or rearrangements.[15,17,18] To maximize mutation detection accuracy in impure clinical specimens, the test was previously optimized and validated to detect base substitutions at a ≥ 5% mutant allele frequency, indels with a ≥ 10% mutant allele frequency with ≥ 99% accuracy, and fusions occurring within baited introns/exons with > 99% sensitivity.[15] Tumor mutational burden (TMB, mutations/Mb) was determined on 0.8 to 1.1 Mbp of sequenced DNA.[18] Microsatellite instability (MSI) was determined on up to 114 loci.[19]

HPV genome sequences were detected by de novo assembly of nonhuman sequencing reads followed by comparison against all viral nucleotide sequences in the National Center for Biotechnology Information (NCBI) RefSeq database using the Basic Local Alignment Search Tool for nucleotides (BLASTn). The RefSeq database is comprehensive and distinguishes HPV types including HPV6, 11, 16, 18, 26, 30, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, 87, CP6108, and IS39. HPV types identified in this study were stratified according to

the HPV classification of Muñoz et al, with HPV16, 18, 31, 33, 51, and 59 labeled high-risk for causing cancer and HPV6 and 11 labeled low-risk.[20] HPV30 and 87 were classified as undetermined risk.[21] HPV positivity was designated for cases with assembled contiguous sequences (contigs) of viral genomic DNA ≥80 nucleotides in length with ≥97% sequence identity to the Refseq sequence identified by BLASTn. Within the HPV51 type, specific sublineages (A1-A4, B1-B2) were identified using short regions that contain lineage-specific and sublineage-specific single nucleotide polymorphisms.[22]

Patient ancestry was determined by classifying specific single nucleotide variations by genomic profiling based on their known variation among populations in the 1000 Genomes Project.[23]

Confirmatory Immunohistochemistry and In Situ Hybridization In-house

We performed confirmatory analyses in-house on all HPV51-positive LMS tumors with available tissue (n = 3). These analyses included immunohistochemistry for desmin (Agilent Technologies, Lexington, MA) and caldesmon (Agilent Technologies), both key markers of smooth muscle lineage. In addition, we completed in situ hybridization for high-risk HPV E6/E7 mRNA using the RNAscope HPV kit (Advanced Cell Diagnostics Inc., Hayward, CA) following the manufacturer's instructions. Formalin-fixed, paraffin-embedded tissue sections were hybridized with a cocktail to detect E6 or E7 mRNA from 18 high-risk HPV types: HPV16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82. Approval for immunohistochemistry and in situ hybridization for these targets, including a waiver of additional informed consent and a HIPAA waiver of authorization, was obtained from the University of California San Francisco Institutional Review Board (protocol No. 11-05569).

Clinicopathologic Assessments of HPV51-positive Leiomyosarcoma Cases

Within our overall cohort of LMS cases, we performed in-depth assessments of the subset harboring HPV51 genomic sequences (HPV51-positive). Clinicopathologic data on the HPV51-positive LMS cases included patient age, sex, tumor site, and FIGO stage or American Joint Committee on Cancer stage (8th edition), which were extracted from accompanying pathology reports.[9,24] Primary site data were available for nearly all cases (91%), with the remaining case designated "indeterminant primary." Histopathology of all HPV51-positive cases was re-assessed on routine H&E-stained slides of tissue sections by 2 board-certified pathologists (E.A.W., D.I.L.).

For categorical data, statistical comparisons between HPV51-positive versus HPV51-negative LMS cases were performed using the Fisher exact test owing to the size of the cohort. For continuous variables (age and TMB), comparisons between the 2 groups were performed using the nonparametric Mann-Whitney *U* test. A 2-tailed *P*-value of <0.05 was considered statistically significant.

RESULTS

Comprehensive Genomic Profiling

To address our hypothesis, we began with a pan-LMS analysis to find cases that were *TP53*/*RB1*-wildtype and harbored HPV genomic sequences. From the entire 2569 LMS cohort, 486 LMS were both *TP53*-wildtype and *RB1*-wildtype (486/2569; 18.9%). From the entire cohort, 48 unique LMS cases carried detectable HPV genomic reads

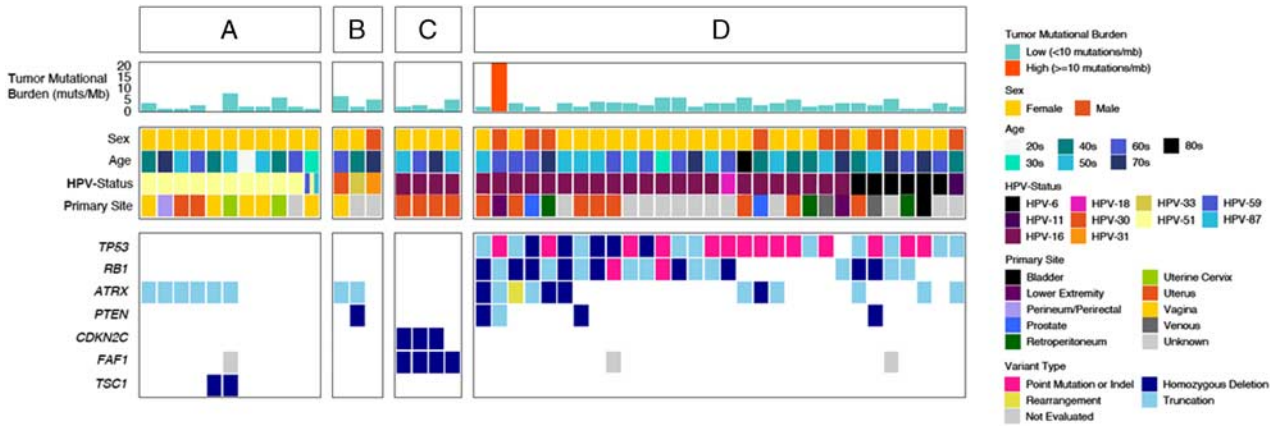

FIGURE 1. Summary tile plot of oncogenic variants in key genes in all leiomyosarcoma cases in which HPV genomic sequences were detected (n = 48/2,569; 1.9%). Here, cases were split into 4 groups from left to right (A–D) based on genomic signature, with HPV the likely tumor driver in group A and potentially B, and HPV genomic reads presumed to be an incidental finding in groups C and D. All 11 cases of HPV51-positive leiomyosarcoma (A) were *TP53/RB1*-wildtype with no detected alterations in *CDKN2C* or *FAF1*, as were 3 additional cases harboring 3 specific non-HPV51 genotypes (B). Next, 4 HPV51-negative, *FAF1*-null cases are shown, 3 of which were also *CDKN2C*-null (C). The remaining 30 HPV-positive LMS cases were all HPV51-negative and harbored *TP53* and/or *RB1* mutations (D). Each column represents data for a single unique patient. Age, sex, HPV status, and tumor primary site are also provided for each case. The histogram on top shows tumor mutational burden (mutations/megabase).

(n = 48/2569; 1.9%; Fig. 1). Overlap between these groups consisted of 18 LMS cases that were *TP53*-wildtype, *RB1*-wildtype, and HPV-positive (Figs. 1A–C). Surprisingly, most of these cases (n = 11) were HPV51-positive (Fig. 1A), and these 11 represented all HPV51-positive tumors in our entire LMS database. HPV51-positive LMS comprised 2.3% of all *TP53*/*RB1*-wildtype LMS (n = 11/486). A single HPV51-positive case had concurrent reads of additional HPV types 59 and 87 (Fig. 1A, rightmost case). All HPV51-positive LMS contained regions that matched to HPV51 sublineage A1, except the single case with concurrent non-HPV51 reads, which matched to HPV51 sublineage B1.

The distribution of GAs in all HPV-positive LMS tumors in our database is displayed in Figure 1, and GAs for all LMS cases are shown in Table 1. HPV51-positive LMS cases (n = 11) were genomically distinct from HPV-positive but HPV51-negative LMS cases (n = 37; Fig. 1A vs. Figs. 1B–D, especially Fig. 1A vs. the 34 cases in Figs. 1C, D) and from all HPV51-negative cases (n = 2558; Table 1), with most of this last group negative for any HPV sequences, as just noted. In particular, *TP53* and *RB1* GAs were strikingly absent from all of the HPV51-positive LMS tumors (Fig. 1A), in contrast to the high prevalence of *TP53* and *RB1* GAs in HPV-positive but HPV51-negative LMS cases (n = 37; Figs. 1B–D; $P < 0.00001$ and 0.001, respectively) and in all of the HPV51-negative LMS (n = 2558; Table 1; $P < 0.00001$ and 0.0002, respectively). The only recurrent pathogenic GAs in HPV51-positive LMS were in *ATRX* and *TSC1*, and these GAs were significantly enriched in HPV51-positive LMS compared with HPV51-negative LMS (Table 1; $P = 0.027$ and 0.0047, respectively). No HPV-positive LMS contained any detectable pathogenic alterations in *PIK3CA*.

Among the HPV51-negative LMS cases, ones with versus without other HPV sequences showed considerable genomic similarities, e.g., GAs in *TP53* were 78% (29/37, Figs. 1B–D) and 72% (1810/2521), respectively; GAs in *RB1* were 57% (21/37) and 53% (1338/2521), respectively; and cases that were both *TP53*-wildtype and *RB1*-wildtype were 18.9% (7/37, Figs. 1B–D) and 18.6% (468/2521), respectively. These similarities suggest that HPV sequences other than HPV51 in our LMS cohort may be merely incidental findings. Importantly, this pattern highlights the contrast between the distinctive genomic signatures of HPV51-positive (Fig. 1A, Table 1) versus HPV51-negative (Figs. 1B–D, Table 1) LMS cases.

Median TMB for HPV51-positive LMS was 1.6 mut/Mb (range <0.8 to 9.6; Q1-Q3 = 0.8 to 2.4), similar to the remainder of the LMS cohort (median = 2.4 mut/Mb; range <0.8 to 203; Q1-Q3 = 1.6 to 4.0) (Table 1; $P = 0.50$, Mann-Whitney *U* test). Median computational tumor purity for HPV51-positive LMS was 71.5% (range: 46.6% to 99.6%). No cases of MSI high were present in the HPV51-positive cohort, which is not statistically distinguishable from the rate of 0.2% in the HPV51-negative LMS group (Table 1).

Of the 37 LMS cases with non-HPV51 HPV reads, 26 contained HPV16, 6 contained HPV6, and 1 each contained HPV11, 18, 30, 31, and 33 (Figs. 1B–D). Of these 37 cases, only 7 (18.9%) were both *TP53*-wildtype and *RB1*-wildtype (Figs. 1B, C), as noted above. Four of these 7 cases harbored HPV16 reads, of which 3 were *CDKN2C*-null and the remaining case was null for an adjacent gene, *FAF1*, consistent with our prior report

TABLE 1. Demographics and Frequencies of Genomic Alterations in Leiomyosarcomas Stratified by HPV51 Status

	HPV51-positive LMS	HPV-negative LMS	*P*
Number of cases	11	2558	
% female	100% (11/11)	80% (2044/2558)	0.14
Median age (range; years)	54 (23-74)	58 (< 1-89+)	0.16
TMB (Q1-Q3; mut/Mb)	1.6 (0.8-2.4)	2.4 (1.6-4.0)	0.50
% MSI high	0% (0/11)	0.2% (5/2144)	> 0.999
TP53	**0% (0/11)**	**72% (1839/2558)**	**< 0.00001**
RB1	**0% (0/11)**	**53% (1359/2558)**	**0.0002**
ATRX	**55% (6/11)**	**24% (602/2538)**	**0.027**
PTEN	0% (0/11)	16% (406/2558)	0.23
TSC1	**18% (2/11)**	**0.6% (23/2558)**	**0.0047**

For percent values, number of positive cases over number of evaluated cases are included in parentheses. Parameters significantly different between HPV51-positive versus HPV51-negative cases are indicated in bold ($P < 0.05$).

TABLE 2. Clinical Characteristics of Patients With HPV51-positive Leiomyosarcoma

Characteristic	N (%)
No of patients	11
Median age at diagnosis, years	54, range: 23-74
Sex	
Female	11 (100)
Male	0
Ancestry*	
European	6 (55)
African	3 (27)
Ad-Mixed American	2 (18)
Primary site	
Vagina	5 (46)
Uterine cervix	2 (18)
Uterine, not otherwise specified	2 (18)
Perineum/perirectal	1 (9)
Indeterminant	1 (9)
FIGO staging (uterine primary)	
IVA	1 (25)
IVB	2 (50)
Unknown	1 (25)
AJCC staging (nonuterine or indeterminant primary)	
II	1 (14)
IIIA	1 (14)
IV	3 (43)
Unknown	2 (29)

*Ancestry superpopulations as defined by the 1000 Genomes Project.[23]

The "Ad-Mixed American" superpopulation was defined by specific SNPs carried by individuals of Mexican-American, Puerto Rican, Colombian, or Peruvian ancestry.[23]

AJCC indicates American Joint Committee on Cancer.

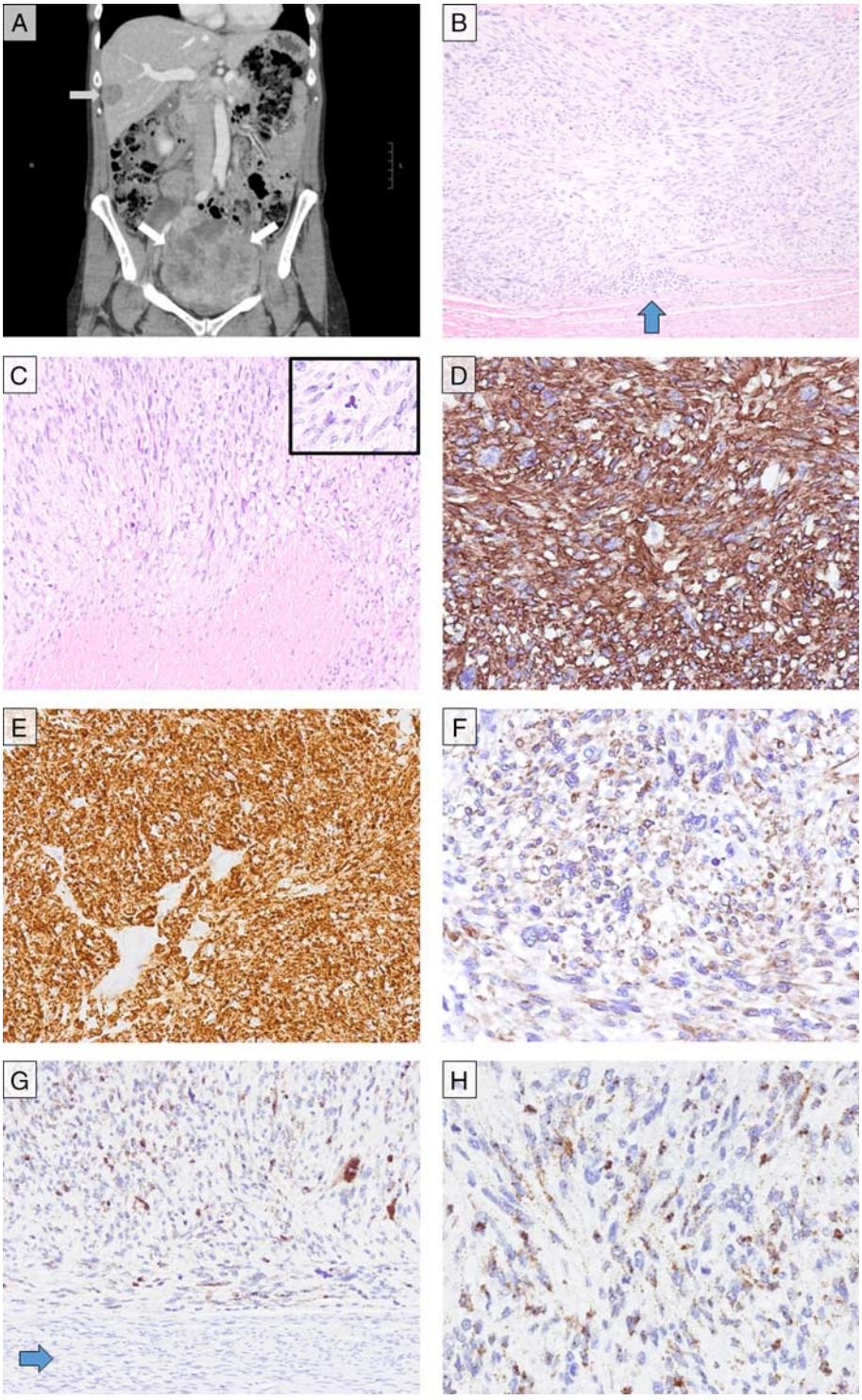

FIGURE 2. Radiology and pathology from patient case 1. A, Abdominopelvic computed tomography with contrast at initial presentation demonstrated a large heterogenously enhancing pelvic mass (white arrows) and a 2.6-cm hypodense mass in the right lobe of the liver (grey arrow). Multiple liver and lung masses were present on different abdominal and thoracic planes on initial imaging (not shown). Histopathologic examination of the pelvic tumor showed fascicles of atypical spindle cells with a pushing border of infiltration (blue arrow) (B; hematoxylin and eosin stain). The tumor also showed areas of geographic necrosis (C) and was mitotically active (C, inset). By immunohistochemistry, tumor cells showed diffuse caldesmon (D), diffuse smooth muscle myosin (E), and scattered desmin positivity (F). By in situ hybridization for high-risk HPV E6/E7 mRNA (detecting 18 HPV genotypes including HPV51), tumors cells showed prominent punctate brown staining, while adjacent nontumoral stroma was negative (blue arrow) (G, H).

TABLE 3. Detailed Clinicopathologic and Molecular Features of All 11 HPV51-positive Leiomyosarcomas

Patient Case	Age (y)/ sex	Primary Tumor Site	Anatomic Location of Sequenced Tumor	Primary Tumor Size (cm)	Predominant Morphology of Sequenced Tumor	Marked Nuclear Atypia	Mitoses/10 HPF
1	56/F	Uterus	Uterus	23.1×15.6×9.3	Spindle	Present	12
2	54/F	Vagina	Vagina	3.0×2.4×1.7	Spindle, with focal myxoid areas	Present	20
3	30/F	Vagina	Vagina	11.0×9.0×5.0	Spindle, with focal myxoid areas	Present	7
4	69/F	CBD	Axillary soft tissue	4.0×3.0×3.0 (axillary mass)	Epithelioid, with clear cell features	Present	11
5	40/F	Vagina	Vagina	CBD	Epithelioid, with focal spindle areas	Present	20
6	47/F	Vagina	Lymph node, station 7	2.5×2.0×1.0	Spindle, with focal epithelioid areas	Present	22
7	74/F	Perineum/ perirectal	Perirectal	CBD	Epithelioid	Present	5
8	23/F	Vagina	Vagina	6.2×5.0×5.0	Spindle, with focal myxoid area	Present	14
9	57/F	Uterine cervix	Lung	CBD	Epithelioid, with focal spindle areas	Present	15
10	66/F	Uterus	Pancreas	CBD	Epithelioid	Present	10
11	41/F	Uterine cervix	Uterine cervix	CBD	Not evaluable owing to crush artifact	Present	Not evaluable owing to crush artifact

Key smooth muscle immunohistochemical markers are indicated in bold.
CBD indicates cannot be determined; F, female.

implicating a central oncogenic role for these gene deletions in a subset of *TP53*/*RB1*-wildtype LMS[25] (Fig. 1C). The remaining three *TP53*/*RB1*-wildtype, HPV-positive, HPV51-negative cases were the ones harboring HPV30, 31, and 33 reads, respectively (Fig. 1B). The *TP53*/*RB1*-wildtype HPV30 and 33 cases were both *ATRX*-mutant (Fig. 1B). Of note, the *TP53*/*RB1*-wildtype HPV30 case was of vaginal primary site. These genomic signatures suggest that the HPV genotypes in Figure 1B could represent mimics of HPV51, but additional cases would be needed to support that inference.

All 6 vaginal primary LMS without HPV reads contained mutations in *TP53*; 4 of these 6 were also *RB1*-mutant, and 2 of the 6 were *ATRX*-mutant.

Clinicopathologic Features of HPV51-positive Leiomyosarcoma

From our cohort of 2569 LMS cases, HPV51-positive LMS accounted for 0.4% of the total (n = 11/2569) and 42% of vaginal primary LMS (n = 5/12), representing a significant enrichment for HPV51-positive cases at this site (42% [5/12] vs. 0.2% of HPV51-negative LMS [6/2557], $P < 0.0001$). Clinical characteristics of HPV51-positive LMS are summarized in Tables 1 and 2. Patients were similar in age and in female-preponderance between HPV51-positive (n = 11) and HPV51-negative LMS (n = 2558; Table 1). Most patients with HPV51-positive LMS in our cohort had clinically advanced disease (Table 2). Anatomic locations and other characteristics of the HPV51-positive tumor specimens that we sequenced are listed in Table 3. Detailed past medical history was available for patient cases 1 and 2 and was unremarkable, other than a history of a cervical cone with cervical intraepithelial neoplasia 3 in patient case 2 that was anatomically discrete from the vaginal mass.

Histopathology

Histopathologic re-evaluation of all 11 HPV51-positive LMS tumors was performed on high-resolution digital path-

TABLE 3. *(continued)*

Necrosis	Border of Infiltration	Vascular Invasion	Immunohistochemistry	HPV E6/E7 mRNA In Situ Hybridization	Genomic Alterations	Disease and Survival Status
Present	Pushing	Not identified	**Caldesmon**: strongly positive **Desmin**: focally positive **Smooth muscle myosin**: strongly positive; CD34/CD117/DOG1: negative ER: negative	Positive	*ATRX* p.E1862fs*17	Dead of disease at 15 mo
Absent	Pushing	Not identified	**Caldesmon**: strongly positive **Desmin**: strongly positive Per report: **Calponin, smooth muscle actin, muscle specific actin**, ER, and PR: diffuse strong positive; Negative for DOG1, CD117, and pan-melanoma; P53: wild-type pattern	Positive	None detected	Disease-free at 84 mo
Absent	CBD	Not identified	**Caldesmon**: focally positive **Desmin**: focally positive Per report: negative for CD34	Positive	None detected	Not available
Absent	Pushing	Not identified	Per report: **Smooth muscle myosin, smooth muscle actin**, and vimentin positive; Negative for S100, MART-1, HMB45, CAM5.2, and AE1/AE3	Not performed	None detected	Not available
Present	CBD	Not identified	Per report: **smooth muscle actin**, **desmin**, vimentin, p16, ER, PR, and p63 positive; Negative for PIN, PAX8, S100, pancytokeratin, CK5/6, CK7, CK20, HMB-45, S100, myogenin, and MyoD-1	Not performed	*ATRX* p.Q2242*, *TSC2* homozygous loss	Not available
Absent	CBD	Not identified	Per report: **smooth muscle actin** positive	Not performed	*ATRX* p.S541*, *CCND3* amplification	Not available
Absent	CBD	Not identified	Per report: **smooth muscle actin, calponin,** and **caldesmon** weakly positive; Negative for SOX-10	Not performed	*ATRX* p.E1065*	Not available
Absent	CBD	Not identified	Per report: ER positive (50% moderate nuclear staining) and PR positive (80% strong nuclear staining)	Not performed	None detected	Not available
Present	CBD	Not identified	Per report: **smooth muscle actin** weakly positive, **desmin** focally positive, and vimentin positive; Negative for pancytokeratin and CK7	Not performed	*ATRX* p.R654*, *TSC2* homozygous loss	Not available
Absent	CBD	Not identified	Per report: negative for chromogranin and cytokeratin cocktail	Not performed	*ATRX* p.R444*	Not available
Present	CBD	Not identified	Not specified on available report	Not performed	None detected	Not available

ology of H&E-stained slides. Histology was heterogeneous, as shown in Figures 2–5 and summarized in Table 3, which includes tumor size, predominant differentiation (spindle, epithelioid, or myxoid), nuclear atypia, number of mitoses, presence of tumor necrosis, border of infiltration and vascular invasion, if known. Overall, HPV51-positive LMS were mitotically active tumors (range: 5 to 22 mitoses per 10 high-power fields, median 14 per 10 high-power fields) with marked nuclear atypia, necrosis, and pushing borders. Five cases had predominant epithelioid morphology and 5 cases were predominantly spindle cell LMS. Predominant morphology of 1 case could not be determined owing to crush artifact (Table 3). Three cases, all vaginal primary cases, showed focal myxoid areas.

Three of 3 HPV51-positive LMS tumors for which in-house confirmatory immunohistochemistry was feasible showed variable positivity for both desmin and caldesmon (Patient cases 1 to 3, Table 3), indicating smooth muscle origin. For 10 of 11 HPV51-positive LMS, findings from immunohistochemistry reports are included in Table 3.

In-house in situ hybridization for high-risk HPV E6/E7 mRNA (detecting 18 HPV genotypes including HPV51) was performed on 3 cases (Table 3), all of which revealed diffuse punctate brown staining of the tumor in each case (Figs. 2G, H, 3D, 4D).

DISCUSSION

Within our cohort of 2569 cases of LMS, HPV51-positive tumors represent a genomically and clinically distinct class of LMS, characterized by the complete absence of detectable in *TP53* or *RB1* mutations, recurrent *ATRX* (55%) and *TSC1* (18%) mutations, the A1 sublineage of HPV51, and a predilection for the female lower reproductive tract as the site of origin. The most common primary site was the vagina, representing a marked enrichment of HPV51-positive cases at this otherwise-rare primary site for LMS. The genomic signature and clinical characteristics of HPV51-positive LMS differed significantly from features of HPV51-negative LMS (Table 1)

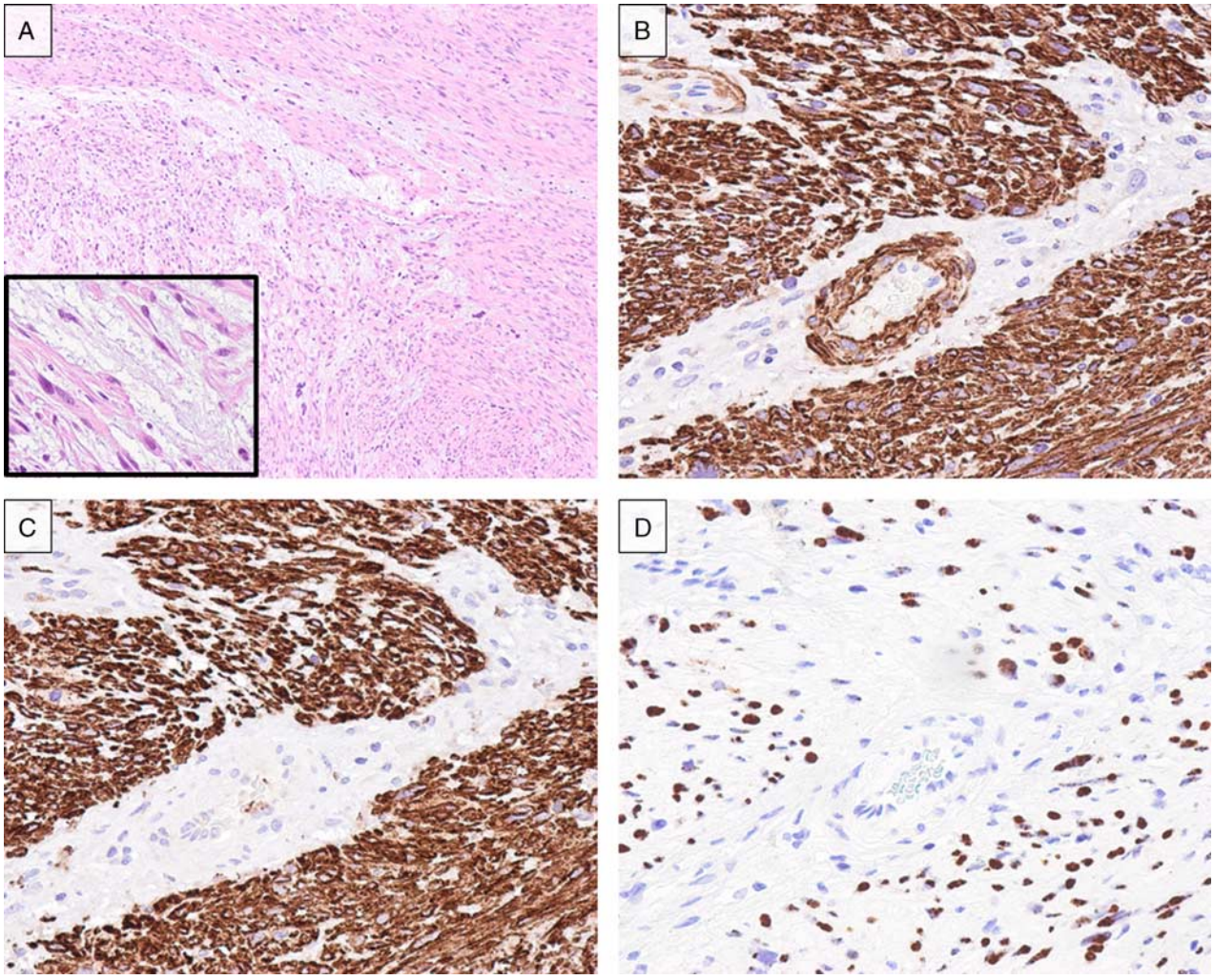

FIGURE 3. Pathology from patient case 2. A, Histopathologic examination of the tumor showed fascicles of spindle cells with focal myxoid areas (inset) (hematoxylin and eosin stain). By immunohistochemistry, tumor cells showed diffuse caldesmon (B) and diffuse desmin positivity (C). By in situ hybridization for high-risk HPV E6/E7 mRNA (detecting 18 HPV genotypes including HPV51), tumor cell showed prominent punctate brown staining, while ad-mixed vasculature was negative (D).

and even from HPV-positive but HPV51-negative LMS (Figs. 1B–D). Thus, although hrHPV types are generally considered pathogenic in carcinomas, our results implicate HPV51, sublineage A1, in the development of this distinct class of sarcoma.

Along similar lines, HPV-positive but HPV51-negative LMS and HPV-negative LMS resembled each other, particularly in their high—and nearly identical—prevalence of *TP53* and *RB1* mutations. Strikingly, no LMS tumor with either *TP53* or *RB1* mutations harbored any detectable HPV51 genomic sequences. Taken together, these data support our hypothesis that HPV can be an alternative tumorigenic mechanism to *TP53* and *RB1* mutations in LMS, specifically implicating HPV51 sublineage A1. Furthermore, we demonstrated direct evidence of high-risk HPV E6/E7 mRNA within the tumor cells of three of three unique HPV51-positive LMS by in situ hybridization (Figs. 2G, H, 3D, 4D), further supporting an oncogenic role of the virus in this new tumor type.

HPV infections have a striking tropism for epithelial cells, so the possibility that these unique lesions represent metaplasia from an epithelial intermediate cannot be entirely excluded. However, for several reasons, we do not favor that these tumors represent carcinomas with mesenchymal differentiation, that is, carcinosarcomas or sarcomatoid/metaplastic carcinomas. First, no areas of epithelial differentiation were identified in any HPV51-positive case on available tissue. Second, while $>30\%$ of HPV-related vulvar squamous cell carcinomas exhibit pathogenic alterations in *PIK3CA*,[14] none of our HPV51-positive LMS contained detectable pathogenic *PIK3CA* alterations. Third, the frequent *ATRX* alterations that we identified in HPV51-positive LMS were not present in a large case series of HPV-related vulvar

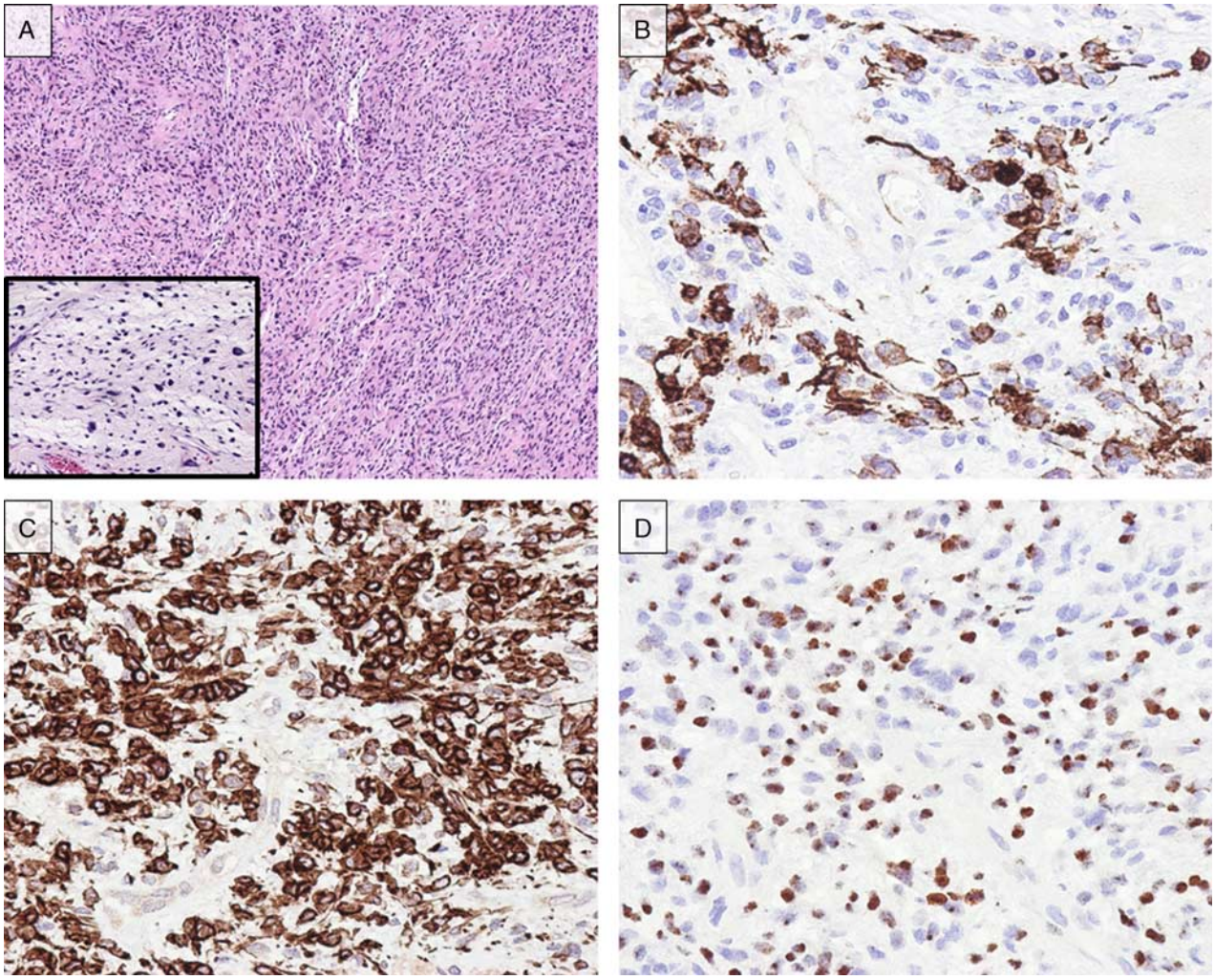

FIGURE 4. Pathology from patient case 3. A, Histopathologic examination of the tumor showed fascicles of spindle cells with focal myxoid areas (inset) (hematoxylin and eosin stain). By immunohistochemistry, tumor cells showed focal caldesmon (B) and focal desmin positivity (C). By in situ hybridization for high-risk HPV E6/E7 mRNA (detecting 18 HPV genotypes including HPC 51), tumor cell showed prominent punctate brown staining (D).

carcinomas,[14] but have been reported as common recurrent genomic events in LMS.[11]

The current study adds a third class of oncogenic mechanisms that may participate in LMS. The first and most common mechanism in LMS involves inactivating mutations in the tumor suppressor genes *TP53* and *RB1* (Fig. 1D, Table 1).[11] Second, we recently reported a role for deletions of *CDKN2C* and the adjacent *FAF1* gene amongst *TP53*-wildtype and especially *RB1*-wildtype LMS, accounting for 3.0% of all LMS cases (Fig. 1C).[25] Here, as a third apparent mechanism, we find a novel role for the HPV51 sublineage A1 in a subset of LMS that are wildtype for both *TP53* and *RB1* (Fig. 1A). This presumptive mechanism is through HPV oncoproteins E6 and E7, which are known to inactivate the p53 and RB1 proteins, respectively.[12] Each of these three distinct classes of molecular participants in different LMS tumors may have therapeutic and prognostic implications that will require further study. A strength of the current study is that we were able to exclude cases with molecular alterations that are considered defining for sarcomas other than LMS.

HPV51 was first described in 1988[26] and is now classified as a hrHPV type.[12,13,27] High-risk HPV genotypes, such as the more frequent HPV16 and HPV18, have been associated mainly with the development of carcinomas, particularly in the cervical, anogenital, and oropharyngeal regions.[12–14] By convention, distinct HPV types are defined by the sequence of the open reading frame that encodes the major capsid protein L1, with a new HPV type defined as differing from all others by at least 10% within this sequence.[22] While different hrHPV types can show different biological activities,[28,29] their oncogenicity results primarily from oncoproteins E6 and E7, as noted above.[12] High-risk oncogenic HPV genotypes are all clustered in one clade composed of species α5, α6, α7, α9, and α11; HPV51 is a member of α5, which consists of types HPV26, HPV51,

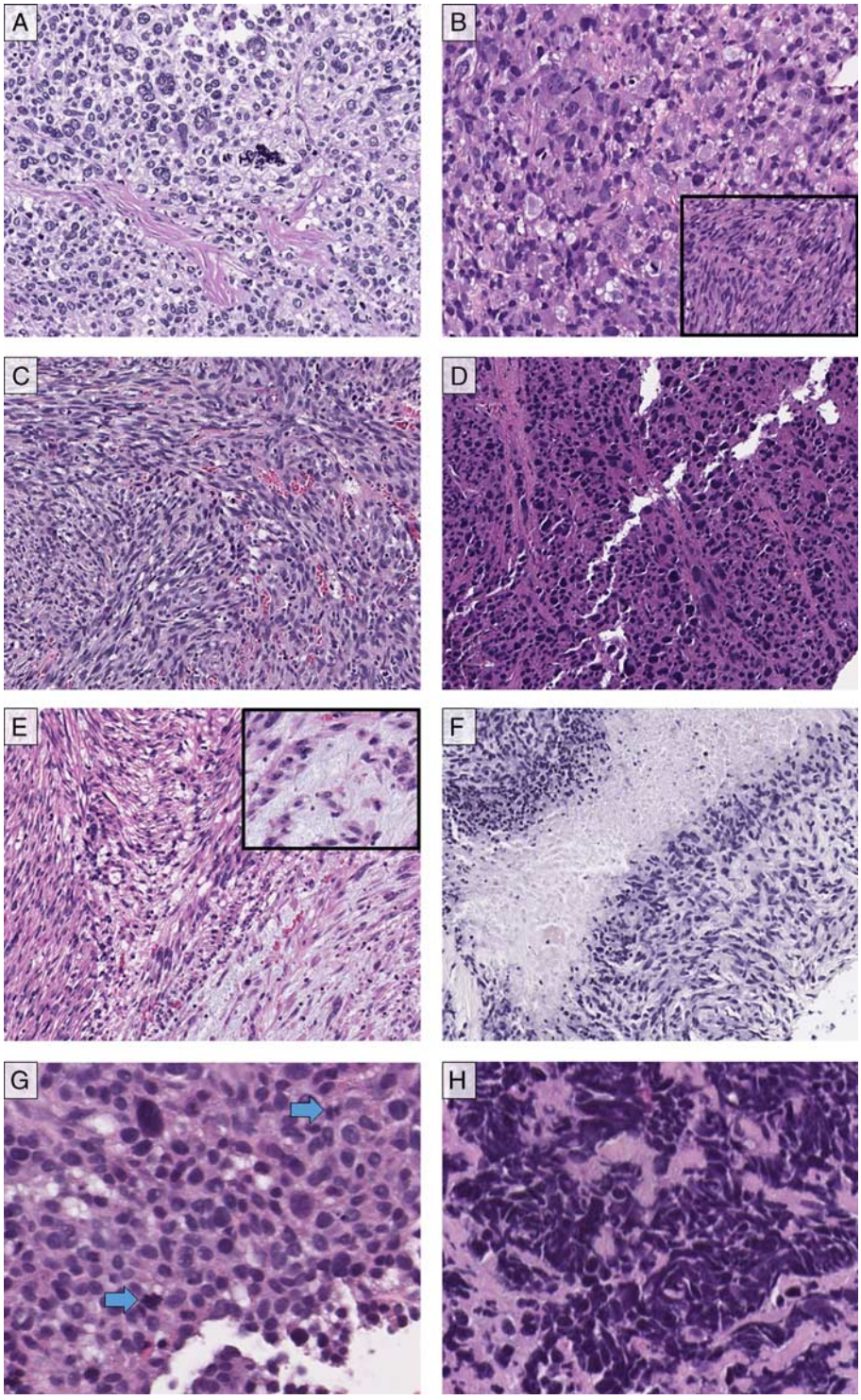

FIGURE 5. Histopathology of the remaining HPV51-associated leiomyosarcomas showed fascicles and sheets of cells that ranged from epithelioid with clear cell features (patient case 4) (A), to epithelioid with focal spindled areas (case 5) (B; inset for spindled area), to predominantly spindled (case 6) (C). Cases also showed marked nuclear pleomorphism and hyperchromasia (case 7) (D) and a confirmed vaginal primary case showed focal myxoid histology (case 8) (E; inset for myxoid material). A subset of cases also showed areas of necrosis (case 9) (F). Higher power examination revealed readily identifiable mitotic figures (blue arrows) and significant nuclear pleomorphism (case 10) (G). A single case showed significant crush artifact, with large cells with markedly elevated nuclear-to-cytoplasmic ratio (case 11) (H) (A–F: H&E stains; G–H: H&E stains).

HPV69, and HPV82.[22] Specific HPV genotype lineages (defined as 1% to <10% genomic differences in the L1 open reading frame) and sublineages (defined as 0.5% to <1% differences), such as HPV16 sublineage D2,[30] have been associated with increased risk of malignancy. HPV51 exhibits considerable genomic variability[31–33] and contains sublineages A1-A4 and B1-B2.[31] No previous study has linked any specific HPV51 lineages to increased cancer risk or to a specific cancer type. For 10 of 11 of the HPV51-positive LMS in our cohort, HPV51 sequences matched to sublineage A1. In a large study of HPV-positive cervical swabs, sublineage A1 was likewise the most common HPV51 sublineage, accounting for 73% of HPV51 infections,[34] but was not linked to any subsequent cancers.

The discovery of HPV as a viral carcinogen led to the development of effective vaccines. Nevertheless, HPV51 is not directly targeted by current vaccines. Data remain inconclusive on cross-protection, that is, the development of immunity to HPV51 after immunization with vaccines that do not specifically target HPV51. One study analyzed several trial cohorts of the HPV 16/18 vaccine, and the data suggested cross-protection against HPV51-associated cervical intraepithelial neoplasia.[35] Additional studies, however, readily identified new HPV51 infections in vaccinated women, with 1 series indicating that HPV51 was the most frequent HPV type identified in new infections (1.38/100 person-year).[36,37]

Importantly, the prevalence of HPV51 differs widely amongst different populations, with particularly high HPV51 infection rates in developing countries.[38–40] Percentages of HPV51 infections range from 3.0% (Jilin province, China) to 31.4% (rural Brazil) and 30.7% (Adama Town, Ethiopia) of hrHPV-positive cervical smears, depending on the population.[38–40] Unfortunately, high rates of nonvaccine hrHPV infections may translate into nonvaccine-related advanced tumors.[41] Of note, 46% of HPV51-positive LMS in our cohort were in individuals of African or ad-mixed American ancestry (Table 2).[23]

HPV51 in routine cervical smears and in neoplasia has frequently been detected in co-infection with other HPV types.[42–44] One recent study identified that biopsies of cervical intraepithelial neoplasia grade 2 to 3 that are infected with HPV51 often show co-infection with other HPV genotypes, with only 15/79 (19%) of HPV51-positive cases showing infection with no other HPV type.[42] The authors of that study concluded that it was unknown whether the inclusion of HPV51 in a new vaccine would improve protection for cervical neoplasia.[43] In contrast, in our cohort of HPV51-positive LMS, HPV51 was the sole HPV genotype identified in 10 of 11 cases (90.9%).

To our knowledge, although incidental HPV has been previously identified in some sarcoma biopsy specimens,[45] no HPV type has been linked to sarcoma by in situ studies. In models of sarcoma in vitro and in animals, hrHPV types 16 and 18 have been shown to transform smooth muscle cells and fibroblasts.[46–50] HPV E6 and E7 proteins have also been identified in smooth muscle cells of aortic tissue and coronary arteries with atherosclerosis[51,52] although their pathogenic effects, if any, in those locations remain unknown. In a few reports, HPV genomic sequences were occasionally identified in rare carcinomas with mesenchymal differentiation, but these were not true sarcomas, and HPV51 was not identified in any of them.[53–58] Uncommon hrHPV genotypes, particularly HPV33, have also been previously identified in a rare cancer type with a characteristic location and histology—namely, HPV-related multiphenotypic sinonasal carcinoma. This distinct sinonasal neoplasm shows characteristic myoepithelial differentiation, frequent surface epithelium involvement, and occasional descriptions of sarcomatoid differentiation,[59] but not a true sarcoma.

Primary vulvovaginal LMS is rare, with an aggressive course and a high recurrence rate.[3–5] A possible link to myxoid histology has been proposed,[60–62] and in our study, HPV51-positive LMS with focal myxoid areas were from only vaginal primary sites and accounted for 3 of the 5 vaginal primary HPV51-positive LMS (Table 3). A substantial percentage of confirmed vulvovaginal primary GYN LMS in our cohort were HPV51-positive (5/12; 42%). An additional single separate vaginal primary case from our cohort was HPV30-positive, a rare HPV type of undetermined risk.[21] Our study may provide insights into the tumor biology of LMS arising from this rare primary tumor site. A strength of our study is that we provide details of clinical, microscopic, and available immunohistochemical parameters, as well as photomicrographs of each of the 11 key cases, plus detailed genomic data, for comparison to guide future studies. If clinically indicated, HPV51 testing, such as by clinically available RNA in situ hybridization (Figs. 2G, H, 3D, 4D), may be warranted for malignant smooth muscle tumors of the lower female reproductive tract or in metastatic LMS of unknown origin, to aid the determination of site of origin.

We identified a few additional HPV genotypes in other rare LMS: in our cohort, nearly all cases with HPV reads that were not HPV51 harbored *TP53*/*RB1* mutations (Fig. 1D) or were *CDKN2C*-null (Fig. 1C),[25] suggesting that the viral reads identified in these cases were an incidental finding and not a driver of tumorigenesis. Supporting this interpretation, we found no statistically significant differences in oncogenic mutational signatures between HPV-positive but HPV51-negative LMS cases versus HPV-negative LMS cases. In addition, no predilection for the female lower reproductive tract was identified in HPV-positive but HPV51-negative LMS cases. In contrast, the single HPV30, 31, and 33 cases in our LMS cohort were *TP53*/*RB1*-wildtype (Fig. 1B) and may therefore represent mimics of HPV51, but additional LMS cases with those 3 HPV genotypes would be needed before inferring an oncogenic role.

Limitations of this study include its retrospective nature and the enrichment for aggressive tumors, mostly metastatic to distant sites, which presumably reflects collection bias from submission of specimens later in the course of disease. In addition, we reviewed H&E scanned slides and accompanying pathology reports from all cases, and we directly reviewed immunohistochemical slides on 3 of 3 evaluable cases shown in Figures 2–4. For other *TP53*/*RB1*-wildtype, HPV51-positive cases, we relied on submitted diagnoses without in-house immunohistochemical confirmation, which is a study limitation. Lastly, confirmatory detection of HPV mRNA by

in situ hybridization was available for three tumors from three different patients of the 11 cases in which we identified HPV51 genomic sequences; 3 of 3 of those tumors contained detectable HPV E6/E7 mRNA.

Additional studies will be needed to determine if the presence of HPV51 in LMS affects prognosis and approaches or responses to treatment. Nevertheless, it is tantalizing to speculate that HPV51-associated GYN LMS may respond to immunotherapy despite low TMB and absence of MSI, similarly to HPV-positive cervical and head and neck carcinomas. If clinical relevance is confirmed, testing of all vaginal primary LMS by clinically available in situ hybridization to find HPV51 genomic sequences may be indicated. Future studies of interest also include characterizing the gene expression profiles of HPV51-associated LMS tumors,[63,64] in comparison with LMS tumors that show mutations in *TP53* and/or *RB1*[11] or deletion of *CDKN2C*.[24] Taking our findings into consideration, it will also be important to resolve whether HPV51 should be included in new vaccines to prevent HPV51-associated LMS and possibly other tumors.

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