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A panel of antibodies to determine site of origin and malignancy in smooth muscle tumors

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Leiomyosarcomas are malignant smooth muscle tumors that occur most commonly in the gynecologic tract and soft tissue. There are different diagnostic criteria of malignancy for smooth muscle tumors arising at gynecologic and soft tissue sites and they may be managed differently but determining the primary site of a smooth muscle tumor can be difficult in some cases. In addition, the distinction between malignant and benign gynecologic tract smooth muscle tumors on morphologic grounds can be challenging. Using a series of tissue microarrays that contain 245 cases of leiomyosarcomas (102 gynecologic) with survival data, and 49 cases of uterine leiomyoma, we examined the ability of selected immune-markers (estrogen receptor (ER) and WT1) to distinguish between leiomyosarcomas of gynecologic and nongynecologic origin. In addition, we examined whether immunostains for p16, p53 and Ki-67 could distinguish between malignant and benign gynecologic smooth muscle tumors. ER nuclear positivity was observed in 3 and 50% of the nongynecologic and gynecologic leiomyosarcomas, respectively ($P<0.001$). Nuclear WT1 positivity was seen in 0 and 8% of the nongynecologic and gynecologic leiomyosarcomas, respectively ($P<0.001$). 87% of primary gynecologic leiomyosarcomas and 2% of uterine leiomyomas showed diffuse ($\geqslant 50\%$ of cells) p16 staining ($P<0.001$). 23% of gynecologic leiomyosarcomas showed p53 immunopositivity ($\geqslant 50\%$ of cells) whereas none of the leiomyomas were positive for p53 ($P<0.001$). 65% of the gynecologic leiomyosarcomas and 0% of the leiomyomas exhibited $>10\%$ Ki-67 proliferation index ($P<0.001$). Diffuse p16 and p53 immunopositivity and high Ki-67 proliferation index, singly or in combination, yielded an overall sensitivity of 92% and specificity of 98% for distinguishing between gynecologic leiomyosarcomas and leiomyomas and can be used as indicators of malignancy for gynecologic smooth muscle tumors. Although ER positivity can be used to support the gynecologic origin of a leiomyosarcomas, nuclear WT1 immunostaining is of little use.
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Leiomyosarcomas are malignant mesenchymal tumors that can occur in the gynecologic tract, soft tissue compartment or visceral sites. Leiomyosarcomas can be broadly grouped into gynecologic type and nongynecologic type based on their anatomic site of origin. Despite the fact that gynecologic and nongynecologic smooth muscle tumors are derived from similar appearing cells, there are significant differences in the criteria used to predict behavior. For example, it has long been recognized that benign behaving smooth muscle tumors of the gynecologic tract can exhibit increased mitotic activity whereas the presence of similar mitotic activity in either deep or superficial soft tissue smooth muscle tumors would lead to an unequivocal diagnosis of leiomyosarcoma.[1] Clinically, these two groups of malignancies are managed differently by different oncologic disciplines.[2,3] Therefore adequate determination of gynecologic versus nongynecologic origin is crucial but can be problematic, especially in tumors that are

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large and located in the pelvis. The majority of uterine leiomyomas and a variable percentage (25–70%) of uterine leiomyosarcomas express estrogen receptor (ER) and/or progesterone receptor (PR).[4–11] This suggests that at least a subset of uterine leiomyosarcomas may originate from ER/PR expressing myometrial smooth muscle cells.[12] In contrast, soft tissue and cutaneous leiomyosarcomas are believed to be derived from vascular smooth muscle and pilar smooth muscle, respectively, both of which lack intrinsic ER/PR expression. ER and WT1 are proposed immunomarkers for identification of primary site of leiomyosarcoma, but their utility has not been assessed in a large series of cases[4–11,13–15] with the largest series containing 38 cases.

Another issue of concern, especially for tumors arising in the gynecologic tract, is that it can be quite challenging to determine whether a smooth muscle tumor will behave in a benign or malignant manner.[16] Significant mitotic activity, focal degenerative cellular atypia and/or hyaline necrosis can all be present in benign uterine leiomyoma. This can be particularly difficult when only a limited biopsy tissue sample is available for assessment. Several immunomarkers, most notably p16, p53 and Ki-67, have been examined previously as aids to distinguish between gynecologic leiomyosarcomas and leiomyomas.[7,11,17–23] However, only small numbers of cases (<50 cases) have been examined in each study, typically with a single marker. In addition, the appropriate diagnostic cut-points for use of these immunomarkers have not been critically evaluated.

In this study, we used a panel of antibodies to address two issues that complicate the diagnosis of smooth muscle tumors and determined cut-points for each marker. First, we examined the utility of ER and WT1 immunomarkers in differentiating between gynecologic and nongynecologic leiomyosarcomas, using a series of 245 leiomyosarcomas. We also assessed the utility of p16, p53 and Ki-67 immunomarkers, alone or in combination, as adjuncts to predict clinical behavior in gynecologic smooth muscle tumors, as tested on 90 primary gynecologic leiomyosarcomas and 49 uterine leiomyomas.

Materials and methods

Case Selection and Tissue Microarray Construction

Paraffin-embedded samples of 245 leiomyosarcomas from 245 different patients were collected from several hospitals and laboratories across the United States, Canada and the Netherlands (Table 1). A total of 149 cases were used previously in studies determining prognostic significance of macrophages[24] and CSF1-associated genes in leiomyosarcomas.[25] Of the 245 leiomyosarcoma tumor samples, 195 were biopsy or resection specimens of the primary tumor, whereas the remaining 50 cases represented biopsy or resection specimens from recurrent (local or distant) disease. Clinical characteristics were available for 221 cases and disease-specific survival (minimum follow-up of 6 months) were available for 74 cases of gynecologic leiomyosarcomas and 64 cases of nongynecologic leiomyosarcomas. For the primary tumors, none of these patients received neoadjuvant treatment (chemotherapy and/or radiotherapy). All of the cases were centrally reviewed (CHL, IE) and the diagnosis of leiomyosarcoma was confirmed based on histologic and immunohistochemical evaluation as described previously.[24] The tumor cell grade was assigned based on the FNCLCC (Fédération Nationale des Centres de Lutte Contre le Cancer) grading system for all leiomyosarcomas. A total of 49 cases of uterine leiomyoma were also obtained from Vancouver General Hospital (Vancouver, BC, Canada) for comparison. The studies were performed with the approval from the institutional review boards at Vancouver General Hospital and Stanford University Medical Center.

Tissue microarrays were constructed as described previously using a manual tissue arrayer (Beecher Instruments, Silver Spring, MD, USA).[24] For each leiomyosarcoma or uterine leiomyoma, a block with more cellular proliferative region of the tumor was chosen; scarred, hyalinized, myxoid or hypocellular areas were avoided. The final tissue microarrays were constructed with one 0.6 mm tissue core and duplicate 0.6 mm tissue cores for individual cases of leiomyosarcoma and uterine leiomyoma, respectively.

Immunohistochemical Staining and Evaluation

Antibodies that target ER, WT1, p16, p53 and Ki-67 were used in the current study. The details of the primary antibodies and the dilutions used are shown in Table 1. Antigen retrieval was performed using CC1 antigen retrieval buffer (Ventana Medical Systems, Tucson, AZ, USA) for all sections.

Table 1 List of the primary antibodies

	Primary antibodies	*Dilutions*
ER	Anti-ER rabbit monoclonal antibody (clone SP1). Lab Vision, Fremont, CA, USA	1:50
WT1	Anti-WT1 mouse monoclonal antibody (clone 6F-H2). Dako, Carpinteria, CA, USA	1:100
p16	Anti-p16^{INK4a} mouse monoclonal antibody (clone 16P04). Cell Marque, Hot Springs, AR, USA	1:20
p53	Anti-p53 mouse monoclonal antibody (clone DO-7). Dako, Carpinteria, CA, USA	1:400
Ki-67	Anti-Ki-67 rabbit monoclonal antibody (clone SP6). Lab Vision, Fremont, CA, USA	1:200

Following incubation with the primary antibodies, sections were stained on the Ventana automated slide stainer (NEXES) using the Ventana diaminobenzidine detection kit (Ventana Medical Systems). Ventana amplification kit (Ventana Medical Systems) was also used for ER immunohistochemistry. For ER, positivity was defined as any positive nuclear staining in $\geq 5\%$ of tumor cells. For WT1, positive nuclear staining was defined as the presence of any nuclear staining in $\geq 5\%$ of tumor cells and positive cytoplasmic staining was defined as the presence of any cytoplasmic staining in $\geq 5\%$ of tumor cells. For p16, positivity was defined as any positive cytoplasmic and/or nuclear staining in $\geq 50\%$ of tumor cells. For p53, positivity was defined as moderate to strong positive nuclear staining in $\geq 50\%$ of tumor cells. Ki-67 staining was evaluated both visually (CHL) and by automated morphometric analysis. For visual pathologist assessment, the percentage of positive nuclear staining in the tumor cells is quantified by visual estimation to the nearest 1% based on assessment of 100 tumor cell nuclei for each core. An average percentage was derived if duplicate cores are present for a given case.

Automated Ki-67 Nuclear Staining Morphometric Analysis

Details of the morphometric scoring were described previously.[26] Briefly, digital images of sections from the TMA stained with Ki-67 were used for morphometric analyses, captured using a BLISS scanner (Bacus Laboratories, Lombard, IL, USA). The slides were scanned at $\times 200$ objective magnification. A custom-written nuclear stain analyzer software ImageJ (http://www.gpec.ubc.ca/index.php?content=software/imagej_plugin/index.php) was used, with the parameters set to optimally identify tumor nuclei. The number of nuclei in each core and its corresponding optical quality were registered. The output data showed the number of brown-stained nuclei (positive staining) and the number of blue-stained nuclei (negative staining) for each core and the percentage of positively stained nuclei was calculated for each core.

Statistical Analysis

Scoring results were combined using Deconvoluter and TMA-Combiner programs (http://genome-www.stanford.edu/TMA/download.shtml). Kaplan–Meier analysis was used to show survival curves with log-rank test to compare survival between two (or more) different groups (WinSTAT 2007). Unpaired Student's *t*-test was used for comparison of the demographic data wherever appropriate (Excel 2007). A *P*-value of less than 0.05 was considered significant.

Results

Patient Demographics

The patient demographic information is shown in Table 2. The 245 leiomyosarcoma samples consisted of 195 primary tumors (105 nongynecologic and 90 gynecologic leiomyosarcomas) and 50 recurrent tumors (38 nongynecologic and 12 gynecologic leiomyosarcomas). The overall age distribution was slightly younger for patients with gynecologic leiomyosarcomas compared to patients with nongynecologic leiomyosarcomas (49 *vs* 53 years, $P=0.16$). Eighty-nine (90%) of the primary gynecologic leiomyosarcomas in our series arose from the uterus whereas the remaining cases occurred in the vagina, adnexa and vulva. Eighty-six (82%) nongynecologic leiomyosarcomas were based in

Table 2 Clinical and pathologic features of leiomyosarcomas

	Gynecologic leiomyosarcoma	*Nongynecologic leiomyosarcoma*
Total number of cases	102	143
Primary	90	105
Recurrence (local/distant)	12	38
Age at time of diagnosis (years)		
Average[a]	49	53
≤40	11	21
41–50	39	34
51–60	40	30
>60	7	39
Sex[b]		
Male	NA	68
Female	102	59
Primary tumors		
Gynecologic Tract ($n=90$)		
Uterus	81	NA
Vagina	3	NA
Adnexa	4	NA
Vulva	2	NA
Nongynecologic tract ($n=105$)		
Retroperitoneum/abdomen/pelvis	NA	40
Soft tissue, limbs	NA	29
Trunk	NA	12
Genitourinary system	NA	7
Thorax	NA	5
Bone	NA	5
Head and neck	NA	3
Others	NA	4
Recurrent tumors		
Local recurrence	7	22
Distant recurrence	5	16
Lung	4	12
Liver		2
Others	1	2

[a]Age at time of diagnosis was not known for 5 gynecologic and 19 nongynecologic LMS cases.
[b]Sex of the patient was not known for 18 nongynecologic LMS patients.
NA: not applicable.

the deep soft tissue of the trunk, limb and body cavity, whereas the remainder occurred in sites such as the genitourinary tract, head and neck region, and bone. Of the 50 recurrent tumors, 29 were from local recurrences whereas 21 tumors represented metastatic tumors, with lung (16 of 21 metastatic tumors) being most common site of distant spread for both gynecologic and nongynecologic leiomyosarcomas. Of the 49 uterine leiomyomas, there were 2 cellular variants, 1 symplastic variant and 2 uterine leiomyomas showing focal infarction, whereas the remainder displayed usual histologic appearance.

ER and WT1 Immunohistochemistry in Primary and Recurrent Leiomyosarcomas and Uterine Leiomyomas

The results of ER and WT1 immunostaining for the 245 leiomyosarcomas and 49 uterine leiomyomas are depicted in Figure 1 with representative images showing the patterns of immunostaining in Figure 2. For gynecologic leiomyosarcomas, ER immunopositivity (defined as nuclear staining in $>5\%$ of tumor cells) was present in 50% (51 of 102 cases) of primary or recurrent gynecologic leiomyosarcomas, with 7 of the 12 recurrent tumors being positive for ER. In contrast, only 3% of nongynecologic leiomyosarcomas (4 of 140 scorable cases) showed positive ER nuclear staining ($P<0.001$). Among the four ER-positive nongynecologic leiomyosarcoma cases, one occurred in the male genital region, one in the male rectal region and two in the female abdominal/pelvic region. For the last two cases, a primary gynecologic site could not be excluded with certainty. All 49 uterine leiomyomas showed positive ER staining.

For WT1, nuclear staining and cytoplasmic staining were assessed separately. Only 8% (8 of 98 scorable cases) of gynecologic leiomyosarcomas showed weak-to-moderate nuclear staining whereas none of the nongynecologic leiomyosarcomas showed detectable nuclear staining ($P<0.001$). The nuclear staining in the positive cases was predominantly weak in intensity (Figure 2). Positive cytoplasmic WT1 immunostaining (cytoplasmic staining in $>5\%$ of tumor cells) was observed in 55% (54 of 98 scorable cases) of gynecologic leiomyosarcomas and 52% (68 of 131 scorable cases) of nongynecologic leiomyosarcomas ($P=0.60$). In contrast to the intensity of nuclear staining, cytoplasmic staining was generally moderate to strong in intensity (Figure 2). 67% (33 of 49 cases) and 90% (44 of 49 cases) of uterine leiomyomas exhibited positive nuclear and cytoplasmic WT1 immunostaining, respectively. All of the cases that exhibited nuclear staining also showed cytoplasmic staining concurrently.

p53, p16 and Ki-67 Immunohistochemistry in Primary Tumors

The results of p53 and p16 immunostaining for the 195 primary leiomyosarcomas and 49 uterine leiomyomas are depicted in Figure 3 with representative images showing the patterns of immunostaining in Figure 4. Positive p53 immunostaining (nuclear staining in at least 50% of tumor cells) was found in 23% (20 of 87 scorable cases) of primary gynecologic leiomyosarcomas whereas none of the 49 uterine leiomyomas showed positive p53 immunostaining ($P<0.001$). 17% (17 of 96 scorable cases) of primary nongynecologic leiomyosarcomas showed positive p53 staining.

Positive p16 immunostaining (nuclear and/or cytoplasmic staining in $\geqslant 50\%$ of tumor cells) was present in 87% (77 of 89 scorable cases) of primary gynecologic leiomyosarcomas and 2% (1 of 48 scorable cases) of uterine leiomyomas ($P<0.001$).

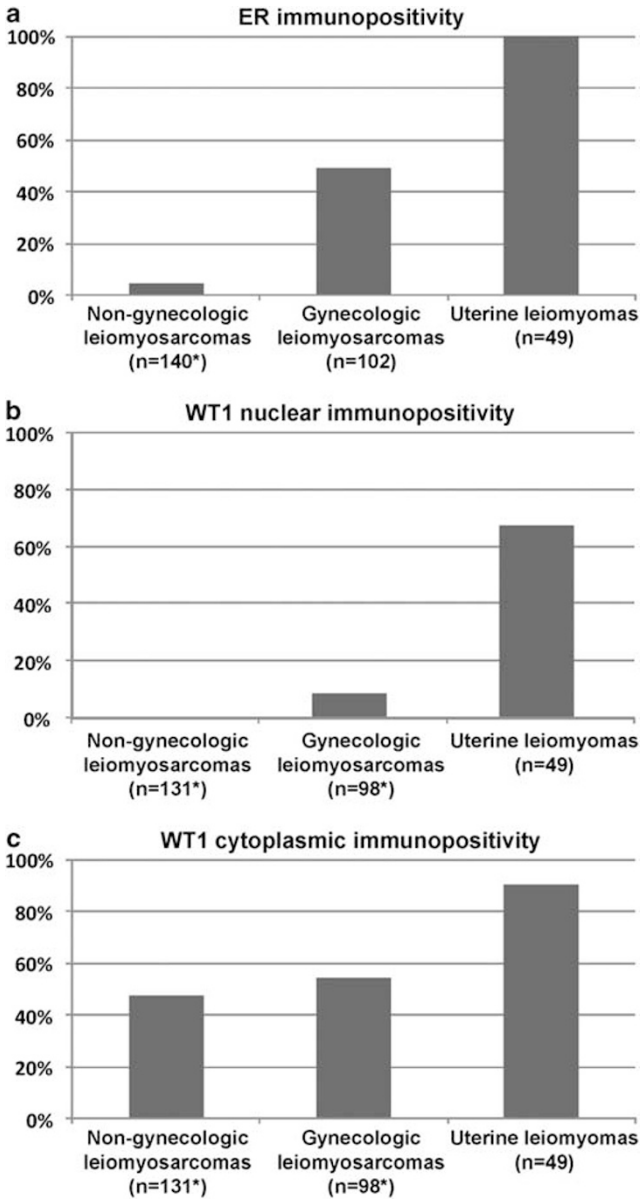

Figure 1 ER (**a**) and WT1 (**b** and **c**) immunostaining patterns in gynecologic and nongynecologic smooth muscle tumors (*, number of scorable cases).

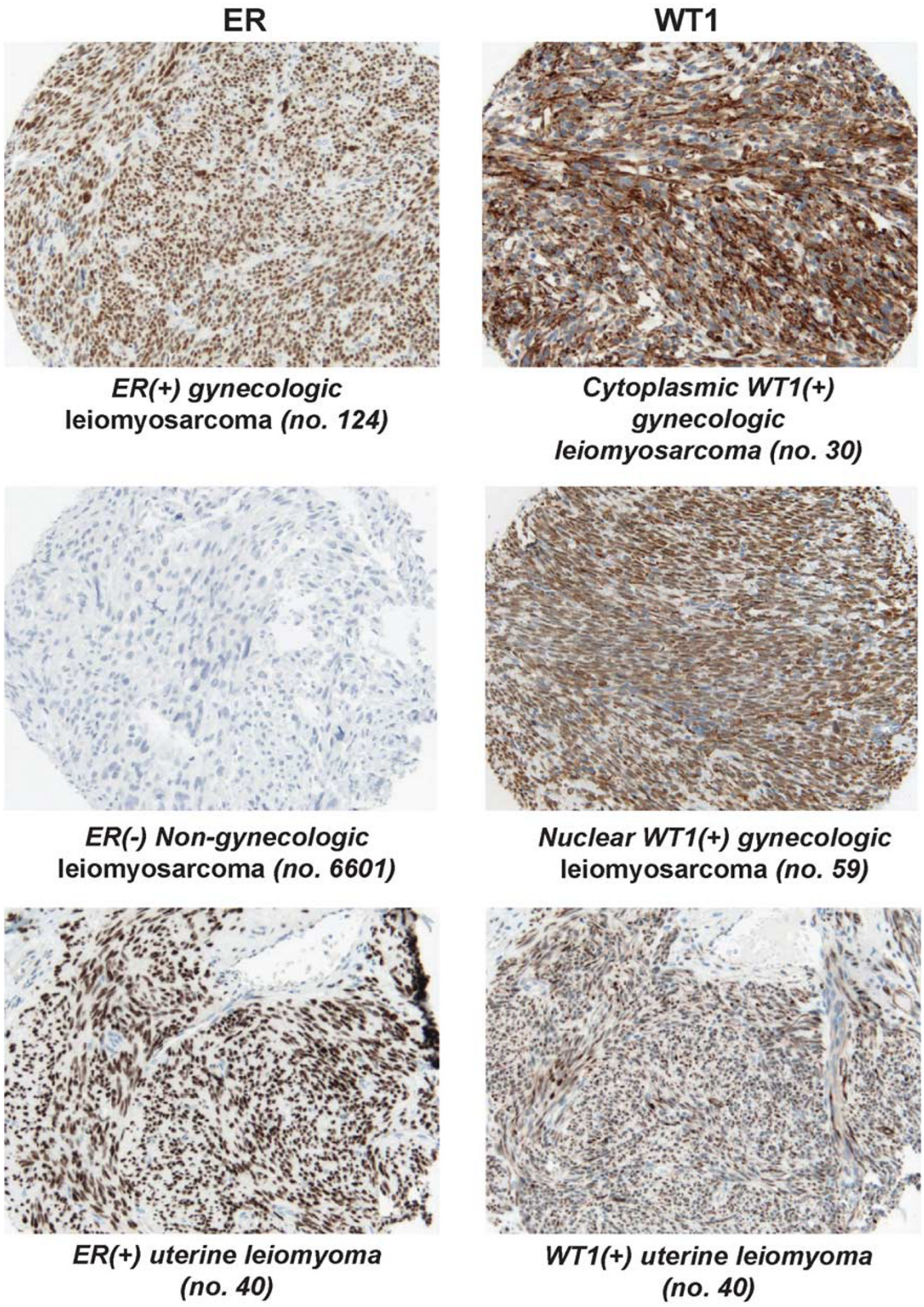

Figure 2 Representative images of ER and WT1 immunostaining in gynecologic and nongynecologic smooth muscle tumors.

The majority (46 of 48 scorable cases) of uterine leiomyomas showed only focal (0–20%) p16 staining. A single case of uterine leiomyoma demonstrated weak cytoplasmic/nuclear p16 staining in 60% of tumor cells and it showed focal hyaline necrosis but no atypia or increased mitotic activity

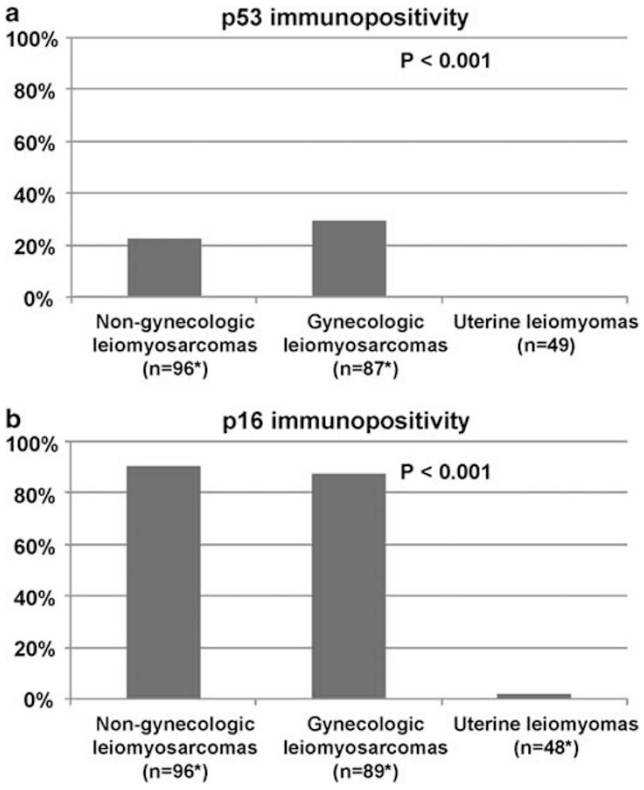

Figure 3 p53 (**a**) and p16 (**b**) immunostaining patterns in primary gynecologic and nongynecologic smooth muscle tumors (*, number of scorable cases).

(Ki-67 staining of 1% by visual analysis and 0.55% by automated analysis). In comparison, 90% (86 of 96 scorable cases) of primary nongynecologic leiomyosarcomas were p16 positive. The positive cases displayed both nuclear and cytoplasmic immunostaining (Figure 4).

Ki-67 immunostaining was evaluated by both visual and automated methods as described in the Materials and methods section, with the results depicted in Figure 5. Based on the findings of prior studies[4,7,11,19,22,23,27–32] a cutoff of 10% was used for both the visual and the automated analyses. The results of manual and automated analyses showed a correlation coefficient of 0.77. Positive Ki-67 staining was observed in 64% (50 of 78 scorable cases) of primary gynecologic leiomyosarcomas and 0% (0 of 49 cases) of uterine leiomyomas by automated analysis ($P<0.001$). Visual analysis yielded near-identical results with positive Ki-67 staining observed in 65% (51 of 78 scorable cases) of primary gynecologic leiomyosarcomas and 0% (0 of 49 cases) of uterine leiomyomas ($P<0.001$). A total of eight cases showed discrepant results between the automated and visual analyses with four cases assessed to be positive by visual method but not by automated method and four cases assessed to positive by automated method but not by the visual method. All the eight cases showed positive p16 immunostaining. By automated analysis, uterine leiomyomas have an average proliferative index of 1.6% (range from 0 to 8.3%) and primary gynecologic leiomyosarcomas has an average of 23.4% (range from 0.1 to 86.1%). The highest proliferation index displayed by any of the uterine variants or uterine leiomyomas with focal hyaline necrosis, was 3.1%. For primary nongynecologic leiomyosarcomas, 67% (53 of 79 scorable cases) and 71% (56 of 79 scorable cases) were found to exhibit positive Ki-67 immunostaining by automated analysis and manual analysis, respectively.

The overall immunostaining results are illustrated graphically in Figure 6. Positivity with at least one of p16, p53 or Ki-67 immunomarkers was seen in 81/88 (92%) of gynecologic leiomyosarcomas, compared to 1/48 (2%) of uterine leiomyomas (sensitivity 92%, specificity 98%). Results of the current study are compared to previously published findings summarized in Table 3.

Kaplan–Meier Survival Analysis of Immunostaining Results

Disease-specific survival data, with a median follow-up period of 3 years, were available for 74 of 90 primary gynecologic leiomyosarcomas and 64 of 105 primary nongynecologic leiomyosarcomas. The Kaplan–Meier survival curves for gynecologic and nongynecologic leiomyosarcomas are depicted in Supplementary Figure 1 ($P=0.73$). Nongynecologic leiomyosarcomas showed an estimated 5-year disease-specific survival of 70%. In contrast, gynecologic leiomyosarcomas showed an estimated 5-year disease-specific survival of below 60% and the survival curve continues to exhibit a downward trend even toward the latter portion of the 5-year period. There was no significant difference in survival based on Ki-67 labeling index, comparing cases with greater than or less than 10% proliferation index, for either gynecologic leiomyosarcomas ($P=0.61$) or nongynecologic leiomyosarcomas ($P=0.23$) (Supplementary Figure 1). No significant association with outcome was found when the median of the Ki-67 proliferation index was used to subdivide the gynecologic or nongynecologic leiomyosarcomas (data not shown). For ER and p53, there were no significant associations observed between disease-specific survival and the immunostaining findings (Supplementary Figure 2).

Discussion

We have used the largest series of smooth muscle tumors examined to date to assess the utility of ER and WT1 in differentiating between gynecologic and nongynecologic leiomyosarcomas, and the utility of p16, p53 and Ki-67 in differentiating between gynecologic leiomyosarcomas and uterine leiomyomas. The findings confirm the results of previous

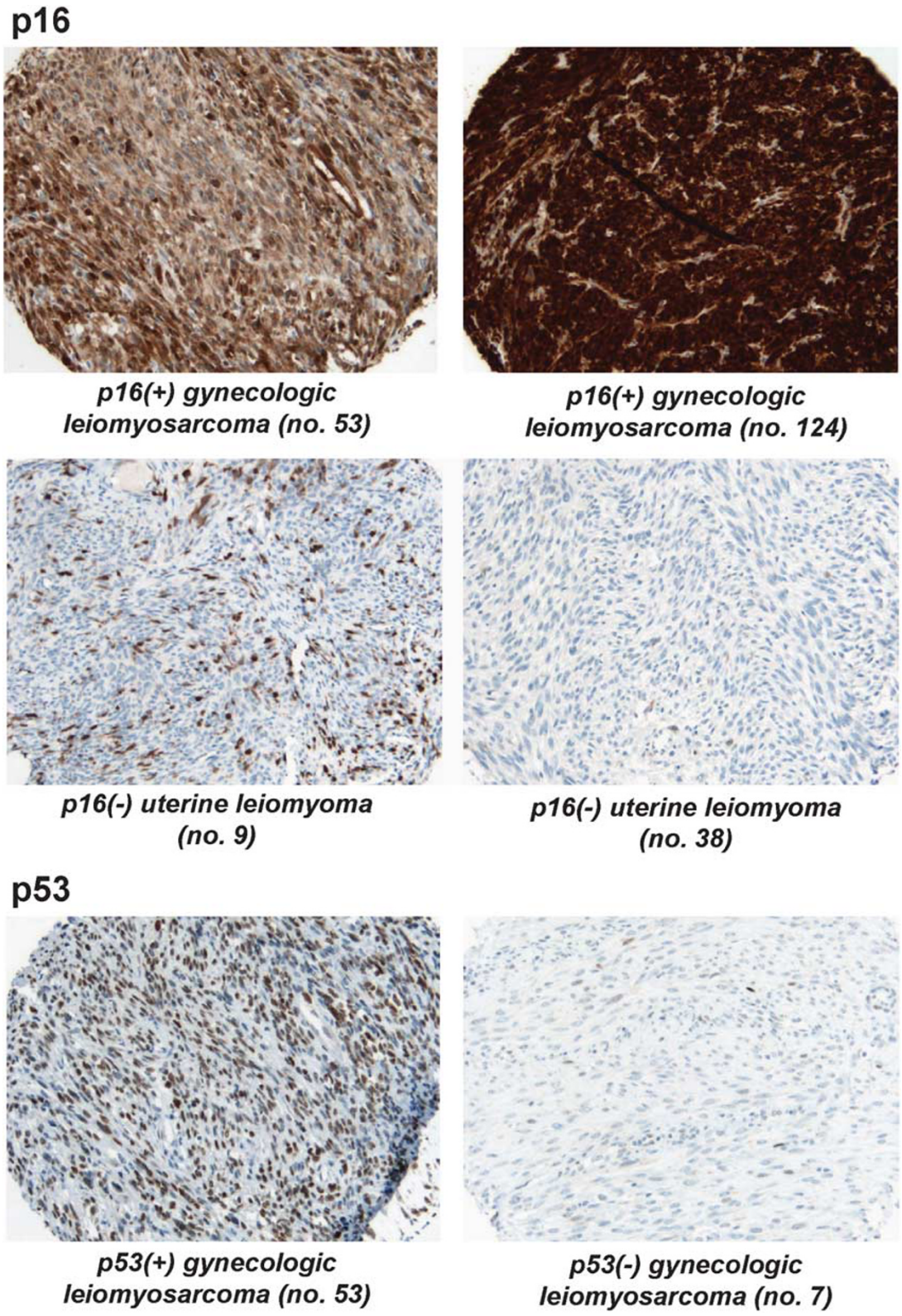

Figure 4 Representative images of p53 and p16 immunostaining in gynecologic and nongynecologic smooth muscle tumors.

studies and demonstrate the value of ER immunostaining in differentiating between leiomyosarcomas of gynecologic and nongynecologic origins and the utility of combined p16, p53 and Ki-67 panel in differentiating between malignant and benign gynecologic smooth muscle tumors.

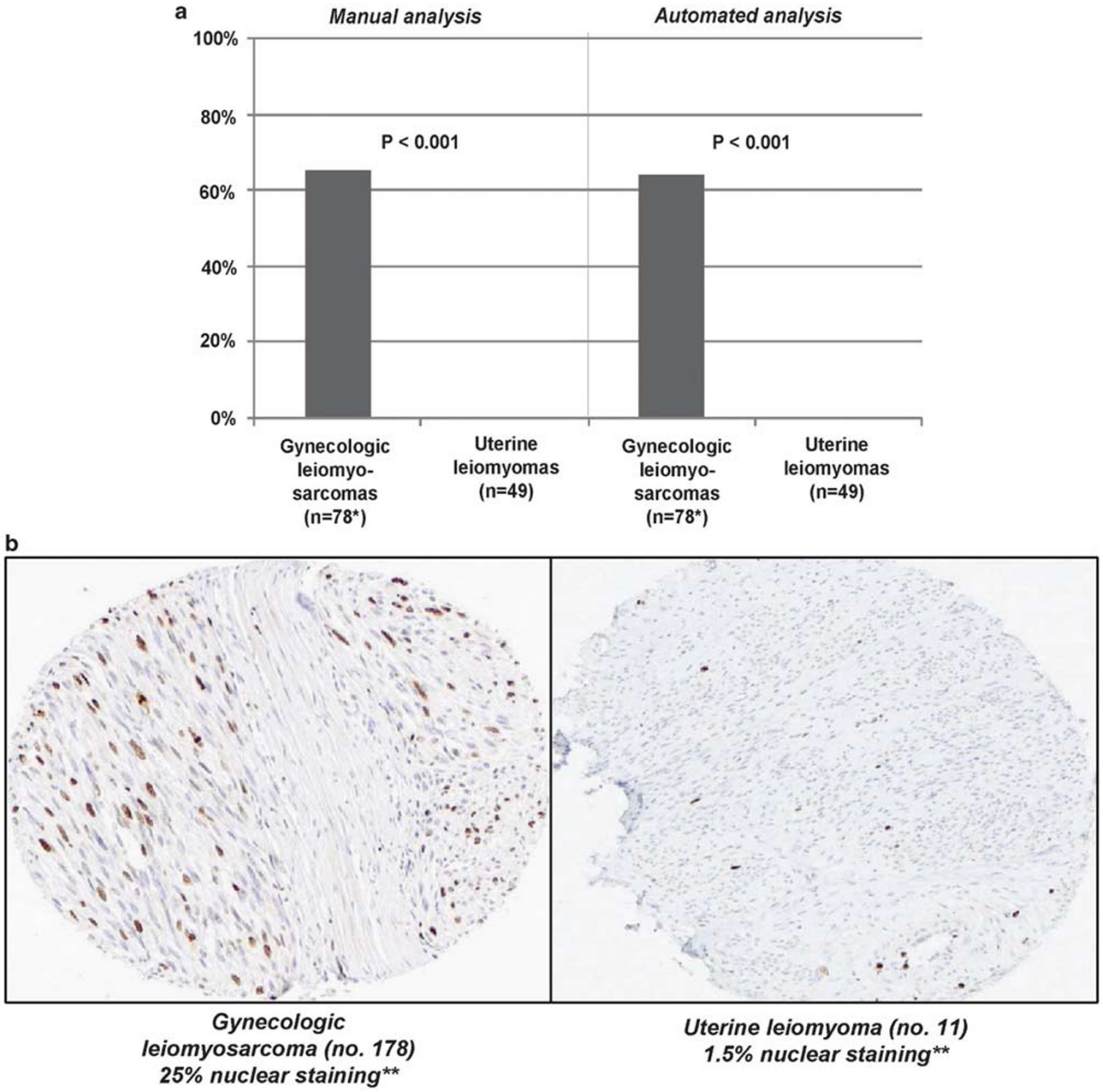

Figure 5 (**a**) Ki-67 immunostaining patterns in primary gynecologic and nongynecologic smooth muscle tumors (*, number of scorable cases). (**b**) Representative images of Ki-67 immunostaining results (**, percent nuclear staining determined by automated morphometric analysis).

Adjunct Markers to Differentiate between Leiomyosarcomas of Gynecologic and Nongynecologic Origin

Two immunomarkers—ER and WT1—were examined for their utility in differentiating between leiomyosarcomas of gynecologic and nongynecologic origins. ER was found to be differentially expressed by gynecologic leiomyosarcomas (50% ER positive) and nongynecologic leiomyosarcomas (3% ER positive), such that the presence of ER immunoreactivity in leiomyosarcoma from either primary or metastatic sites would strongly favor a gynecologic origin with a specificity of 94%. The combined results of previous studies (for 129 cases) showed a very similar finding with 52% ER positivity in gynecologic leiomyosarcomas.[4–11] Using a cutoff of 5% tumor cell nuclear staining, Rao *et al*[10] reported ER positivity in 6% of nongynecologic leiomyosarcomas, a finding similar to our current observation. In contrast, Kelly *et al*[8] used a lower cutoff of 1% and observed ER positivity in 4 of 16 (25%) nongynecologic leiomyosarcomas, with 3 of the 4 positive cases from female

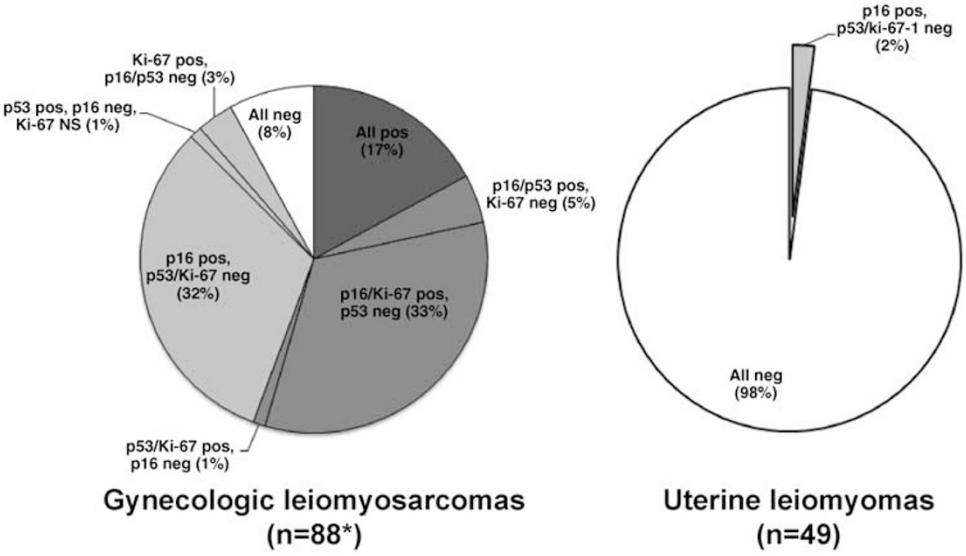

Figure 6 Summary of the immunohistochemical staining results in gynecologic leiomyosarcomas and uterine leiomyomas (pos, positive; neg, negative; NS, nonscorable; *, number of scorable cases).

patients. Setting a cutoff at 5% may allow for better discrimination between gynecologic and nongynecologic leiomyosarcomas. In support of this notion, we have observed from unpublished gene expression profiling study that 9 of 17 (53%) gynecologic leiomyosarcomas but only 1 of 26 (4%) nongynecologic leiomyosarcomas show significant ESR1 expression levels (unpublished data).

As to WT1, 8% of the gynecologic leiomyosarcomas and 0% of nongynecologic leiomyosarcomas showed positive nuclear staining whereas 55% of gynecologic leiomyosarcomas and 52% of nongynecologic leiomyosarcomas showed positive cytoplasmic staining. This finding is different from that reported by Deavers *et al*[15] both qualitatively and quantitatively. Using the same antibody targeting WT1 (Dako clone 6F-H2), Deavers *et al*[15] reported, in a series of 20 cases, positive nuclear WT1 staining in 60% of gynecologic leiomyosarcomas and in 0% of nongynecologic leiomyosarcomas. Two other previous studies have also assessed immunohistochemical expression of WT1 in gynecologic and nongynecologic leiomyosarcomas using the same anti-WT1 antibody.[13,14] The findings from these two studies are more comparable to our current findings as they reported predominantly cytoplasmic pattern of immunostaining in 64–76% of gynecologic leiomyosarcomas with one study reporting predominantly cytoplasmic WT1 immunostaining in 58% of nongynecologic leiomyosarcomas. The reason underlying these discrepant findings is unclear though Deavers *et al* appeared to have used a different secondary antibody detection system based on the abstract that was published.[33] Regardless of the source of this discrepancy our data indicate that WT1 is a less useful marker than ER in determining site of origin for smooth muscle tumors.

Adjunct Markers to Differentiate between Malignant and Benign Gynecologic Smooth Muscle Tumors

Three immunomarkers—p16, Ki-67 and p53—were examined for their utility in differentiating between malignant and benign primary gynecologic smooth muscle tumors. Similar to the reported frequency of p16 positivity in 84% of gynecologic leiomyosarcomas (101 cases from 5 studies), we now observed p16 positivity in 87% of gynecologic leiomyosarcomas in our series. Only 2% of uterine leiomyomas from our series exhibited p16 positivity (positive staining in $\geq$50% of tumor cells), whereas 11 of 108 (10%) combined cases from 5 published studies of uterine leiomyoma showed variable degree of positive p16 staining.[17–20,31] Of these 11 positive cases, 8 originated from studies in which a lower cutoff for p16 positivity (25 and 33%) was used[17–19] and the 3 remaining positive cases came from a study that considered any strong immunostaining of tumor cells to be positive.[20] In general, all of the studies reported the pattern of p16 to be typically focal and weak in uterine leiomyomas, in contrast to the more diffuse and intense pattern of staining seen in leiomyosarcomas. It would appear based on our current finding that the use of a higher threshold value (ie 50%) for p16 can improve the specificity of p16 without significant compromise to its sensitivity.

Positive p53 immunostaining was found in 29% of gynecologic leiomyosarcomas and 0% of uterine leiomyomas in our current series based on a cutoff of

Table 3 Summary of the immunostaining results from the current study and previously published studies

Studies	*Gynecologic leiomyosarcomas*	*Nongynecologic leiomyosarcomas*	*Smooth muscle tumors of uncertain malignant potential*	*Uterine leiomyomas, variant*[a]	*Uterine leiomyomas*	*Criteria for positivity*
ER staining						
Ip *et al*[39]			15 of 15			All >10%
Akhan *et al*[4]	5 of 19					>10%
Leitao *et al*[9]	10 of 25				15 of 19	NA
Kelley *et al*[8]	13 of 15	4 of 16				>1%
Bodner *et al*[18]	12 of 21		13 of 24		24 of 26	>10%
Bodner *et al*[18]	12 of 21					>10%
Zhai *et al*[11]	5 of 14		5 of 8	18 of 18	20 of 20	>5%
Rao *et al*[10]	10 of 14	1 of 16			5 of 5	>5%
Combined published data	*67 of 129 (52%)*	*5 of 32 (16%)*	*33 of 47 (70%)*	*18 of 18 (100%)*	*64 of 70 (91%)*	
Data from current study	*51 of 102 (50%)*	*4 of 140 (3%)*		*3 of 3 (100%)*	*46 of 46 (100%)*	*≥5%*
Overall combined data	*118 of 231 (51%)*	*9 of 172 (5%)*	*33 of 47 (70%)*	*21 of 21 (100%)*	*110 of 116 (95%)*	
WT1 staining						
Bing *et al*[13]	16 of 25	14 of 24				>5%
Deavers *et al*[15]	12 of 20	0 of 22	3 of 3	3 of 3	16 of 16	Any
Coosemans *et al*[14]	29 of 38					Any
Agoff *et al*[40]				9 of 9		NA
Combined published data	*57 of 83 (69%)*	*14 of 46 (30%)*	*3 of 3 (100%)*	*12 of 12 (100%)*	*16 of 16 (100%)*	
Data from current study	*54 of 98 (55%)*	*68 of 131 (52%)*		*3 of 3 (100%)*	*42 of 46 (91%)*	*≥5%*
Overall combined data	*111 of 181 (61%)*	*82 of 177 (46%)*	*3 of 3 (100%)*	*15 of 15 (100%)*	*58 of 62 (94%)*	
p53 staining						
Ip *et al*[39]			2 of 15			>66%
Chen *et al*[19]	32 of 35		1 of 2	9 of 28	0 of 35	>moderate
O'Neill *et al*[31]	8 of 22		0 of 4	2 of 27	0 of 10	>25%
Anderson *et al*[34]	12 of 25				0 of 19	NA
Akhan *et al*[4]	4 of 19					>25%
Mittal *et al*[23]	5 of 12		0 of 7	0 of 15		≥15%
Layfield *et al*[37]	4 of 9				0 of 15	>5%
Zhai *et al*[11]	7 of 14		0 of 8	0 of 18	0 of 20	>5%
Blom *et al*[35]	13 of 49					>10%
Hall *et al*[21]	3 of 23				0 of 20	NA
De Vos *et al*[36]	3 of 8				0 of 8	NA
Amada *et al*[27]	8 of 24			0 of 25		Any
Niemann *et al*[38]	16 of 34		1 of 6		0 of 18	>20%
Jeffers *et al*[29]	13 of 23		6 of 10		1 of 18	NA
Konomoto *et al*[41]		8 of 37				Any
O'Reilly *et al*[32]		19 of 44				Any
Combined published data	*128 of 297 (43%)*	*27 of 81 (33%)*	*10 of 52 (20%)*	*11 of 113 (10%)*	*1 of 163 (0.6%)*	
Data from current study	*25 of 87 (29%)*	*21 of 96 (22%)*		*0 of 3 (0%)*	*0 of 46 (0%)*	
Overall combined data	*153 of 384 (40%)*	*48 of 177 (27%)*	*10 of 52 (20%)*	*11 of 116 (9%)*	*1 of 209 (0.5%)*	
p16 staining						
Ip *et al*[39]			2 of 15			>66%
Atkins *et al*[17]	11 of 15		3 of 8		0 of 23	>33%
Gannon *et al*[20]	8 of 8			7 of 31	3 of 14	Any moderate/ strong
Chen *et al*[19]	35 of 35		2 of 2	18 of 28	5 of 35	>25%
O'Neill *et al*[31]	19 of 22		1 of 4	7 of 27	0 of 10	>50%
Bodner-Adler *et al*[18]	12 of 21		5 of 24		3 of 26	>33%
Combined published data	*85 of 101 (84%)*		*13 of 53 (25%)*	*32 of 86 (37%)*	*11 of 108 (10%)*	
Data from current study	*77 of 89 (87%)*			*0 of 3*	*1 of 45 (2%)*	*≥50%*
Overall combined data	*162 of 190 (85%)*		*13 of 53 (25%)*	*32 of 89 (36%)*	*12 of 153 (8%)*	
Ki-67 labeling index						
Ip *et al*[39]			1 of 15			>10%
Chen *et al*[19]	29 of 35		2 of 2	7 of 28	0 of 35	>10%
O'Neill *et al*[31]	20 of 22		2 of 4	14 of 27	4 of 10	>10%
Akhan *et al*[4]	14 of 19					>10%
Mayerhofer *et al*[22]	10 of 20		0 of 22		2 of 25	>10%
Mayerhofer *et al*[22]	10 of 20					>10%
Mittal *et al*[23]	11 of 12		0 of 7	0 of 15		>10%
Chou *et al*[28]	3 of 6				0 of 44	>10%
Amada *et al*[27]	13 of 24			0 of 25		>10%
O'Reilly[32]		29 of 44				Any
Combined published data	*110 of 158 (70%)*	*29 of 44 (66%)*	*5 of 50 (10%)*	*21 of 95 (22%)*	*6 of 114 (5%)*	
Data from current study	*51 of 78 (65%)*	*56 of 79 (71%)*		*0 of 3 (0%)*	*0 of 46 (0%)*	*>10%*
Overall combined data	*161 of 236 (68%)*	*85 of 123 (69%)*	*5 of 50 (10%)*	*21 of 98 (21%)*	*6 of 160 (4%)*	

NA: not available.
[a]The uterine leiomyoma variant includes cellular leiomyoma and symplastic leiomyoma.
Values in italics are used for summated values.

50% nuclear staining. A large number of studies have previously examined the pattern of p53 immunostaining in gynecologic smooth muscle tumors and cumulatively across 297 cases, p53 positivity was observed in 43% of gynecologic leiomyosarcomas and less than 1% of uterine leiomyomas.[4,7,11,19,21,23,27,29,31,34–38] The lower frequency of p53 positivity seen in our study may be attributed to the significantly lower cutoff value used by several of these earlier studies, whereas the two previous studies that used a more comparable cutoff value of 25% showed a similar rate of p53 positivity ranging from 21 to 36% of the cases.[4,31] Even though the sensitivity of p53 is poor for detecting malignant gynecologic smooth muscle tumors, its specificity appears to be exceptional based on all available data (>99%).

In this study, the percentage of Ki-67-positive nuclear staining was evaluated both visually and by automated morphometric analysis. The results were highly concordant between these two analytic methods, with about two-thirds of primary gynecologic leiomyosarcomas and 0% of uterine leiomyomas showing >10% positive nuclear staining. In comparison, 70% of previously reported gynecologic leiomyosarcomas (158 cases from 8 studies) and 5% of previously reported conventional uterine leiomyomas (114 cases from 6 studies) showed >10% Ki-67 labeling index by visual method of analysis.[4,7,11,19,22,23,27–32] It is unclear whether any of these uterine leiomyomas with elevated Ki-67 labeling index exhibited increased mitotic index.

A limitation of our current study is the paucity of variant uterine leiomyomas and lack of smooth muscle tumors of uncertain malignant potential examined. Only one symplastic and two cellular uterine leiomyomas were included in our current series. Although all three cases demonstrated negative staining for p16 and p53, and were all associated with a low Ki-67 proliferation index, it is certainly of insufficient sample size for meaningful assessment. Our review of the literature showed that a higher percentage of variant uterine leiomyomas (inclusive of symplastic uterine leiomyoma and cellular uterine leiomyoma) and smooth muscle tumors of uncertain malignant potential demonstrate positive p16 or p53 staining, though as noted earlier, these studies used a lower threshold cutoff value for positivity. In addition, 11 and 22% of smooth muscle tumors of uncertain malignant potential and variant uterine leiomyomas, respectively, appear to exhibit greater than 10% Ki-67 nuclear staining by tumor cells.[19,22,23,27,31] However, most of these earlier studies lacked clinical follow-up data necessary for correlation with outcome. Intriguingly, recent studies have shown a tendency for smooth muscle tumors of uncertain malignant potential that behave in a malignant manner, with local or systemic recurrences to exhibit diffuse p16 and/or p53 immunostaining.[17,39] Most recently, Ip *et al*[39] observed the presence of diffuse p16 and p53 positivity in 2 of 16 smooth muscle tumors of uncertain malignant potential examined, both of which developed local recurrence within 5 years after the initial surgery and were the only cases in which the disease recurred in that series. Further studies are needed to evaluate the utility of p16, p53 and Ki-67 in predicting the clinical behavior of these diagnostically challenging gynecologic smooth muscle tumors. Another limitation of the study is that we only evaluated the staining patterns of tissue cores containing representative areas of the tumors and did not assess for potential intratumoral variability on whole sections for each cases. Although the current study helps to establish the staining patterns between benign and malignant smooth muscle tumors, future study looking at staining patterns on whole tissue sections will be needed, particularly focussing on diagnostically challenging cases.

Although not the focus of our current study, we analyzed the ability of our markers to predict outcome in cases diagnosed as leiomyosarcoma. We did not find a significant association between survival and the Ki-67 proliferation index for gynecologic leiomyosarcomas in our current series. In contrast, two studies have previously reported improved survival in patients with gynecologic leiomyosarcomas that show lower proliferation index (using 10 and 30% cutoffs).[4,22] No significant association with disease-specific survival was found in the current series for p53 and ER positivity in gynecologic leiomyosarcomas either.

In summary, we have shown the utility of p16, p53 and Ki-67 in differentiating between malignant and benign tumors in a large series of primary gynecologic smooth muscle tumors. We have also shown the specificity of ER positivity for leiomyosarcomas of gynecologic origin in a large series of primary/recurrent gynecologic and nongynecologic leiomyosarcomas. These findings can help pathologists when faced with these challenging diagnostic scenarios.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Modern Pathology website (http://www.nature.com/modpathol)