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Differential expression of P16 and P21 in benign and malignant uterine smooth muscle tumors

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Abstract
Purpose The diagnosis of benign and malignant uterine smooth muscle tumors depends on morphologic criteria such as nuclear atypia, coagulative tumor cell necrosis and mitotic activity. Most of these tumors are readily classifiable into benign or malignant categories using these criteria. However, the distinction between leiomyomas and leiomyosarcomas may at times be problematic. Hence, it would be useful to have additional markers which could help to distinguish these tumors. The aim of the study was to evaluate p16 and p21 expressions in uterine smooth muscle tumors and determine whether p16 and p21 have a potential value in the differential diagnosis of problematic cases. In addition, we evaluated whether the differential expression of p16 and p21 in uterine leiomyosarcomas correlated with tumor recurrence and patient survival.
Methods p16 and p21 expressions were investigated by immunohistochemistry from paraffin-embedded tissues in 53 cases of uterine smooth muscle tumors consisting of 15 cases of leiomyoma, 14 cases of atypical leiomyoma (leiomyoma with bizarre nuclei), 3 cases of smooth muscle tumor of uncertain malignant potential (STUMP) and 21 cases of leiomyosarcoma. Cases were evaluated with respect to both staining percentage and intensity.
Results There was a statistically significant difference in p16 and p21 staining percentage and intensity between leiomyosarcomas and the other groups. There was no difference in p16 and p21 expressions between leiomyomas, atypical leiomyomas (leiomyoma with bizarre nuclei) and STUMPs. Multivariate analysis showed that the tumor stage was the only independent significant prognostic factor for overall survival in leiomyosarcomas. Neither p16 nor p21 was correlated with disease-free or overall survival.
Conclusions Our findings suggested that p16 and p21 may be of value as an adjunct to conventional morphologic criteria in the assessment of problematic uterine smooth muscle tumors.

Keywords Uterine smooth muscle tumors · p16 · p21 · Immunohistochemistry · Differential diagnosis

Introduction

Smooth muscle tumors are the most common neoplasms of the uterus. Leiomyoma (LM) is the most common type and occurs in nearly 40% of women older than 35 years. Uterine leiomyosarcomas (LMSs) are relatively rare smooth-muscle tumors, accounting for approximately one-third of uterine sarcomas and 1.3% of all uterine malignancies [1]. Most of these tumors are readily classifiable into benign or malignant categories using a combination of microscopic features including the presence and type of necrosis, the degree of cytologic atypia, the mitotic activity, and the relationship of the tumor to surrounding normal structures. However, a small number of uterine smooth

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muscle tumors pose difficult diagnostic challenges. Morphologically, some variants of LM, such as cellular leiomyoma (CLM), atypical leiomyoma (leiomyoma with bizarre nuclei) (ALM) and mitotically active leiomyoma (MAL) can mimic malignancy in one or more respects. Also some smooth muscle tumors that cannot be classified as benign or malignant based on histopathological criteria are diagnosed as the smooth muscle tumor of uncertain malignant potential (STUMP). This term is used when there is significant doubt about the failure rate associated with a particular combination of microscopic features [1]. The ultimate biological behavior of tumors classified as STUMP remains uncertain. Thus, it would be clinically valuable to decrease the percentage of these cases for the optimal management of the patients.

Many studies have used various histochemical and immunohistochemical markers that might be useful in differential diagnoses of uterine smooth muscle tumors, but the difficulties in differential diagnoses cannot be solved yet.

Cellular proliferation is normally regulated by proto-oncogenes and onco-suppressor genes, with opposite functions on cellular growth. The loss of cell cycle control is a critical step in the development of neoplasia. The roles of cell cycle regulators, such as p16 and p21, have been studied in a variety of human neoplasms. In uterine smooth muscle tumors, there are limited data about p16 and p21 expressions [2–11]. The aim of the study was to ascertain whether there are differences in the expressions of p16 and p21 between the various types of uterine smooth muscle neoplasms and whether p16 and p21 have a potential value in the differential diagnoses of problematic cases. In addition, we evaluated whether the differential expression of p16 and p21 in uterine leiomyosarcomas correlated with tumor recurrence and patient survival.

Materials and methods

Tissue collection

This retrospective study included 53 cases of uterine smooth muscle tumors (15 cases of LM, 14 cases of ALM, 3 cases of STUMP and 21 cases of LMS) diagnosed between 1997 and 2006 at the Department of Pathology of the Eskisehir Osmangazi University Medical Faculty. The clinical parameters of the patients such as the age, operation type, postoperative treatment and follow-up information were obtained from the files of Department of Gynecology and Obstetrics of the Eskisehir Osmangazi University Medical Faculty. All hematoxylin and eosin (H&E)-stained slides were reviewed and the diagnoses were confirmed. Microscopic characteristics such as nuclear atypia, mitotic activity, coagulative tumor cell necrosis, cellularity, vascular invasion, and borders with the adjacent tissue were analyzed. Mitotic counts were performed in the most mitotically active areas and the number of mitoses in 10 consecutive high power fields was counted. Necrosis, if found, was categorized as hyaline or coagulative tumor cell necrosis.

Immunohistochemistry

H&E-stained slides from each case were examined to identify the representative area of tumor. Immunohistochemical analysis for p16 and p21 with a labeled streptavidin–biotin–peroxidase complex technique was performed on formalin-fixed and paraffin-embedded tumor sections. Commercially available antibodies against p16 (Cell Marque, USA) and p21 (ScyTek, USA) were used in this study. Only nuclear staining was considered as a positive reaction for p16 and p21. The evaluation of the immunohistochemical staining was done in two ways:

1. *Staining percentage*: The percentage of tumor cells with positive staining was estimated by counting 1,000 tumor cells. Five scores were used to describe the number of positively stained tumor cells: score 0 (none of the tumor cells), score 1 (<10% of the tumor cells), score 2 (10–25% of the tumor cells), score 3 (26–50% of the tumor cells), and score 4 (>50% of the tumor cells).
2. *Staining intensity*: Nuclear staining intensity for each case was graded subjectively from (+) to (+++), with (+) as weak, (++) as moderate, and (+++) as strong staining.

Statistical analysis

Pearson Chi-square exact and Fisher exact tests were used to compare the frequency distributions of p16 and p21 protein expressions between the various uterine smooth muscle tumors. Multivariate Cox proportional hazards regression models were used for survival analysis. *P* values of less than 0.05 were considered statistically significant. Data were stored and analyzed using the SPSS 13.0 statistical software.

The study was performed in accordance with local laws, and approval was obtained from the Ethics Committee of the Medical Faculty, University of Eskisehir Osmangazi, Turkey.

Results

Clinical and pathological features of patients

Among the 53 cases of uterine smooth muscle tumors, 21 of them were diagnosed as LMS, 14 as ALM, 15 as LM,

and 3 as STUMP. Clinicopathologic features of these cases are summarized in Table 1. In all patients with LMS, the tumors contained limited to widespread coagulative tumor cell necrosis. Mitotic index was in excess of 10 MF/10HPF in 17 (80.9%) cases and moderate or severe cytologic atypia was noted in all cases of LMS. Leiomyosarcoma cases were graded according to FNCLCC (Federation Nationale des Centres de Lutte Contre le Cancer) grading system for soft tissue sarcomas [12]. Three (14.28%) patients were grade 1, nine (42.85%) were grade 2, and nine (42.85%) were grade 3. All patients with LMS were staged according to the FIGO staging system for uterine sarcomas [13]. Twelve (57.14%) patients had stage I, one (4.76) patient stage II, five (23.80%) patients stage III and three (14.28%) patients stage IV disease. Two patients with stage IV disease had distant metastases (liver and lung) at diagnosis and one patient had tumoral invasion to bladder and pelviabdominal cavity. During the follow-up period, two other patients developed distant metastases (liver and brain). Recurrence was observed in five patients with a mean recurrence-free survival of 11.8 months (range 3–40 months). Sixteen (76.1%) patients died of disease with a mean overall survival of 20.3 months (range 3–92 months). The diagnosis of STUMP was used in cases in which there was uncertainty about the mitotic index or the type of necrosis. Two cases of STUMP had uncertain type of necrosis and other one had structures that could be regarded either as mitotic figures or smudged karyorrhectic nuclei. In cases with STUMP, follow-up information was available in two of three patients. Both the cases have behaved in a benign fashion with no evidence of recurrence after 36 and 120 months of follow-up. Clinicopathologic features and follow-up data of STUMP cases are summarized in Table 2. All cases diagnosed as LM and ALM have behaved in a benign fashion.

Expression of p16 protein in LM, ALM, STUMP, and LMS

The results of the immunohistochemical staining are shown in Table 3. p16 protein was expressed in 20 (95.2%) cases of LMS and staining percentage was in excess of 10% in 17 cases. Only one (33.3%) case of STUMP showed expression of p16 with a staining percentage of 12% and a staining intensity of (++). Only two (14.2%) cases of ALM showed p16 expression with a staining percentage of 1 and 21% and a staining intensity of (+) and (++). Only five (33.3%) of 15 LM cases exhibited p16 positivity and the staining percentage was less than 10% of tumor cells in all these cases. Representative examples of the immunohistochemical analysis of p16 in patients with STUMP and LMS are shown in Figs. 1 and 2.

Table 1 Clinicopathologic features of the study population

Diagnosis	*n*	Mean age (range)	Mean size (cm) (range)	Nuclear atypia	Mean mitoses (/10HPFs) (range)	Necrosis	Grade	Lymphovascular invasion	FIGO stage	Treatment	Recurrence	Outcome
Leiomyosarcoma	21	58.14 (39–78)	16.75 (4–26)	Moderate, severe, 21	19.6 (2–48)	CTCN 21/21	G1, 3 G2, 9 G3, 9	4/21	I, 12 II, 1 III, 5 IV, 3	TAH-BSO, 21 CT, 2 RT, 3 CRT, 8	5/21	DOD, 16 NED, 5
STUMP	3	45.67 (39–51)	10.00 (3–14)	Mild, moderate, 3	4.33 (2–9[a])	Uncertain type[b], 2 None, 1		0/3		TAH, 3	0/2	NED, 2 LFU, 1
Atypical Leiomyoma	14	45.93 (37–51)	4.33 (1–7.5)	Moderate, severe, 14	2.92 (1–8)	0/14		0/14		TAH, 13 myomectomy, 1	0/14	NED, 14
Leiomyoma	15	47.33 (37–61)	4.32 (1.2–11)	None, 15	1.46 (1–4)	CTCN (0/15)		0/15		TAH, 12 STAH, 3	0/15	NED, 15

BSO bilateral salpingo-oophorectomy, *CT* chemotherapy, *CRT* chemoradiotherapy, *DOD* died of disease, *LFU* lost of follow-up, *NED* no evidence of disease, *RT* radiotherapy, *STAH* subtotal abdominal hysterectomy, *TAH* total abdominal hysterectomy

[a] Case in which 9 MF/10HPF was observed also had structures that could be regarded either as mitotic figures or smudged karyorrhectic nuclei

[b] Coagulative tumor cell necrosis or infarct-type necrosis

Table 2 Clinicopathologic features and follow-up data of STUMP cases

Case	Age	Size (cm)	Cellularity	Nuclear atypia	Mitoses (/10HPFs)	Necrosis	p16 (%)	p16 (intensity)	p21 (%)	p21 (intensity)	Outcome (mo)
1	47	14	Increased	Diffuse mild, focal moderate	9[a]	None	12	++	11	++	NED (120)
2	51	13	Increased	Multifocal, mild–moderate	2	Uncertain type[b]	0	0	1	+	NED (36)
3	39	3	Increased	Multifocal, moderate	2	Uncertain type	0	0	0	0	LFU

NED no evidence of disease, *LFU* lost of follow-up

[a] Case in which 9 MF/10HPF was observed also had structures that could be regarded either as mitotic figures or smudged karyorrhectic nuclei

[b] Coagulative tumor cell necrosis or infarct-type necrosis

Table 3 Staining percentage and staining intensity of tumor cells for p16 protein

	LM ($n = 15$)	ALM ($n = 14$)	STUMP ($n = 3$)	LMS ($n = 21$)
Staining percentage for p16 (%)				
0	10	12	2	1
<10	5	1	0	3
10–25	0	1	1	3
26–50	0	0	0	6
>50	0	0	0	8
Staining intensity for p16				
0	10	12	2	1
+	2	1	0	4
++	3	1	1	3
+++	0	0	0	13

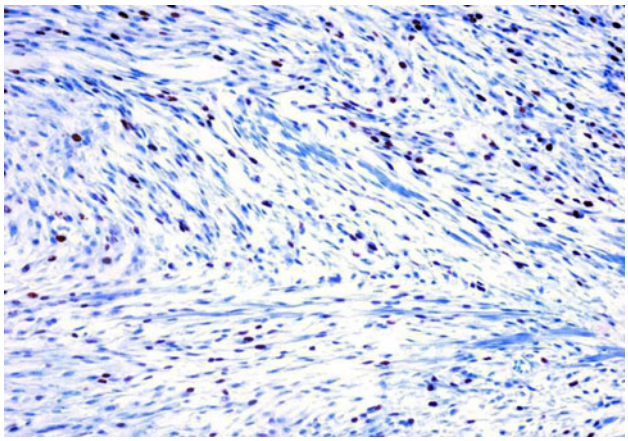

Fig. 1 Scattered nuclear p16 positivity in a STUMP (×100)

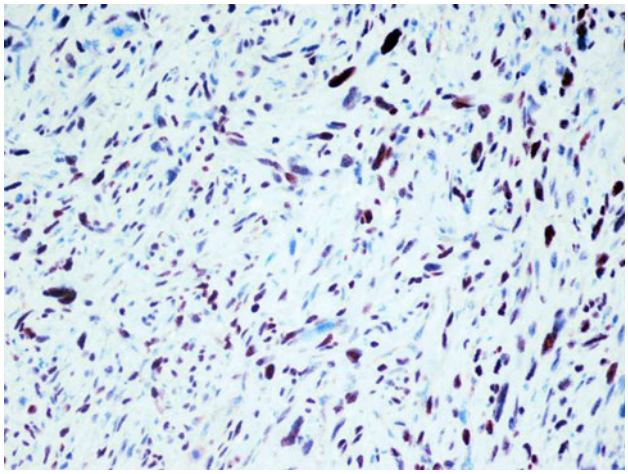

Fig. 2 Diffuse nuclear p16 positivity in a leiomyosarcoma (×200)

Table 4 Staining percentage and staining intensity of tumor cells for p21 protein

	LM ($n = 15$)	ALM ($n = 14$)	STUMP ($n = 3$)	LMS ($n = 21$)
Staining percentage for p21 (%)				
0	10	7	1	0
<10	4	6	1	1
10–25	1	1	1	6
26–50	0	0	0	7
>50	0	0	0	7
Staining intensity for p21				
0	10	7	1	0
+	0	2	1	5
++	4	4	1	6
+++	1	1	0	10

Expression of p21 protein in LM, ALM, STUMP, and LMS

The staining percentage of p21 and the staining intensity in all cases are shown in Table 4. p21 protein was expressed in all LMS cases. The staining percentage was in excess of 10% in 20 (95.2%) cases. Two (66.6%) cases of STUMP showed p21 expression with a staining percentage of 1 and 11%. Seven (50%) cases of ALM showed positivity with a staining percentage of 1–12%. Five (33.3%) of 15 LM cases exhibited positivity with p21 and the staining percentage was less than 10% in 4 cases and 11% in other case. Representative examples of the immunohistochemical analysis

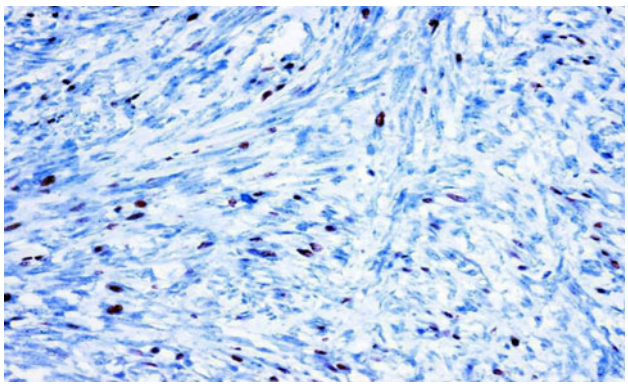

Fig. 3 Atypical leiomyoma showing scattered nuclear p21 expression (×200)

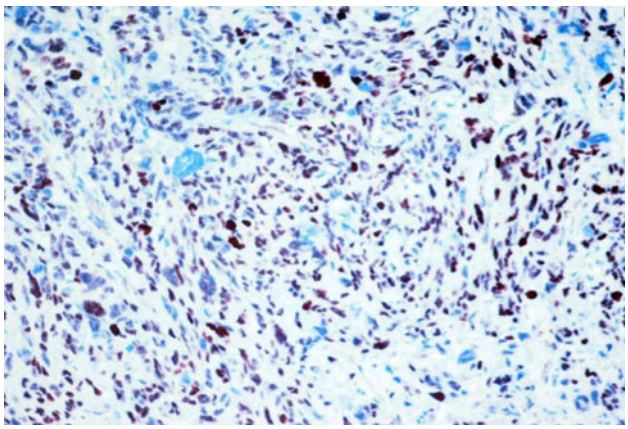

Fig. 4 Diffuse strong nuclear p21 positivity in a leiomyosarcoma (×200)

of p21 in patients with ALM and LMS are shown in Figs. 3 and 4.

There was a statistically significant difference in the staining percentage and intensity with p16 and p21 between LMSs and the other uterine smooth muscle tumor groups (LM, ALM and STUMP) ($P < 0.05$). No statistically significant difference was observed in the staining percentage and intensity with p16 and p21 between LM, ALM and STUMP groups ($P > 0.05$).

Mann–Whitney U and Pearson's Chi-square tests showed that there was no statistically significant difference for staining percentage and intensity of p16 and p21 between LMS cases with low (<10MF/10HPFs) and high (≥10MF/10HPFs) mitotic activity ($P > 0.05$).

Relationship of clinicopathological factors with survival in leiomyosarcomas

The samples were divided into low (≤45%) and high (>45%) p16 expression groups according to the median value. The cut off value of 40% (the median value of p21 expression) divided whole series into the tumors with low (≤40%) and high (>40%) p21 expression. To determine the independent prognostic value relative to disease-free and overall survival, p16 and p21 expression, as well as other clinicopathological parameters such as patient age, tumor size, mitotic count, histological grade, vascular invasion, metastasis, and clinical stage were included in Cox regression model. Multivariate analysis showed that the tumor stage was the only statistically significant independent predictor for overall survival in LMSs ($P = 0.041$). The relative risk of death for patients with stage III and IV disease was 3.177-fold higher than that for the patients with stage I and II disease. No significant correlation was noted between disease-free survival and expressions of p16 and p21, as well as other clinicopathological parameters.

Discussion

Although most of the uterine smooth muscle tumors are readily classifiable into benign or malignant categories using a combination of microscopic features, in occasional cases the distinction between these tumors may be difficult or impossible. Several ancillary techniques have been used for years to accurately classify these tumors into benign and malignant categories**.** But still, there can be difficulties in the differential diagnosis. The role of cell cycle regulators in various neoplasms have been studied widely**.** But there are limited data about their roles in mesenchymal neoplasms. Also the data about p16 and p21 expressions in uterine smooth muscle tumors is limited [2–11].

p16 protein is a cyclin-dependent kinase (CDK)-4 inhibitor and acts as a negative cell cycle regulator. Various studies have shown that the gene coding p16 is deleted in many of the malignant tumors [14–17]. To date, the major diagnostic usefulness of p16 immunohistochemistry has been in the field of gynecologic pathology, not only for prognostic assessment of different tumors but also for differential diagnostic purposes. p16 was found to be useful to distinguish high-grade CIN from atrophic squamous epithelium in postmenopausal vomen [18]. Regauer and Reich [19] showed that immunohistochemistry for p16 and cytokeratin 17 allows the separation of immature metaplasia with or without reactive atypia from CIN III. A panel of antibodies, comprising p16, MIB-1 and bcl-2, was found to be useful adjunct to histology in distinguishing cervical glandular intraepithelial neoplasia from tuboendometrioid metaplasia, microglandular hyperplasia and endometriosis [20]. In the literature, there are limited data about the immunohistochemical expression of p16 in uterine smooth muscle neoplasms [2–9]**.** To date, there is general agreement that p16 is more frequently and more

strongly expressed in LMSs compared to LMs and is a useful antibody in discriminating LMSs from LMs [2–6, 8, 9]. In our series, 20 (95%) of 21 LMSs showed p16 positivity. Seventeen of 21 LMS cases had expression of p16 in more than 10% of the tumor cells. In contrast, all LM cases showed negative or weak (less than 10%) immunostaining for p16. This difference was statistically significant. Our results are in line with the previous reports and suggest that p16 might be a useful immunohistochemical marker in distinguishing uterine LMSs from LMs.

STUMP cases represent the most serious diagnostic problem among uterine smooth muscle tumors. There are conflicting results about the role of p16 expression in distinguishing LMSs from STUMPs. The conflicting results are probably due to the different study populations in terms of clinical behavior, as in some studies all the STUMP cases had a benign outcome, while in others some cases developed recurrence. Part of the discrepancy in the p16 stain results between the studies may also be related to the fact that there is no uniformity in the definition of STUMPs. Bodner-Adler et al. [2] observed that the immunohistochemical profile of STUMP seems to be closer to LM than LMS. Similarly, p16 was found to be overexpressed in uterine LMSs compared STUMPs by O'Neill et al. [3]. On the other hand, Atkins et al. [4] found that three of the eight STUMPs showed strong, diffuse p16 positivity. In the study by Ip et al. [7] consisting of 16 cases of STUMP, 2 patients who developed recurrence had strong p16 expression in >66% of tumor cells, whereas the 5 patients with tumors having <33% positively stained cells were all disease-free at last follow-up. In another study, both cases of STUMP were found to show overexpression (>25% of the tumor cells) of p16 similar to LMSs, but the authors do not mention about the clinical behavior of these cases [5]. Among the three STUMP cases in our study, follow-up information was available in two cases. Only one of three STUMP cases showed expression of p16 in more than 10% of the tumor cells in contrast to LMSs who had p16 positivity in more than 10% of the tumor cells in 17 of 21 cases. This STUMP case behaved in a benign fashion with no evidence of recurrence after 120 months of follow-up. Other case with available follow-up data was free of disease after 36 months of follow-up. But this follow-up interval appears too short, because recently Ip et al. [21] reported an average interval of 51 months for recurrence in STUMP cases. We observed that the STUMP cases showed similar immunohistochemical p16 staining with LMs and that there were statistically significant differences in the staining percentage and intensity with p16 between STUMPs and LMSs. Our findings appear to be not entirely in agreement with those of some other studies. But unlike to our STUMP cases which behaved in a benign fashion, Atkins et al. [4] showed that three of the eight tumors originally classified as STUMP developed metastatic disease and two of these tumors had strong, diffuse p16 positivity. Both cases were classified as STUMP based on coagulative tumor cell necrosis in the absence of significant mitoses or cytologic atypia. The authors concluded that the addition of p16 may aid in discerning a subset of STUMP that should be classified as LMS. In the study of Ip et al. [7], the two recurrent tumors were the only ones that were strongly immunoreactive for p16, suggesting that this marker may be helpful in the prediction of the behavior of STUMPs. In the series of O'Neill et al. [3] one of the four cases of STUMP exhibited 5+ staining (>75% of tumor cells) with p16 and this metastasized to the spinal cord 12 months after removal of the primary uterine neoplasm. Although our data cannot definitely prove the role of p16 in distinguishing STUMPs from LMSs due to the small number of STUMP cases, our study, along with others, suggests that p16 expression may be helpful in predicting the behavior of STUMP cases.

We found that only 1 of 14 ALM cases showed expression of p16 in more than 10% of the tumor cells and there was a statistically significant difference in the staining percentage and intensity with p16 between ALMs and LMSs. In our study, all cases diagnosed as ALM have behaved in a benign fashion. Consistent with our study, Gannon et al. [6] reported that only two of the eight cases of ALM showed a final score of +1 and remaining six cases were negative for p16, whereas p16 staining was identified in all eight LMS cases at either +2 (4/8) or +3 (4/8) overall staining intensity. When comparing ALMs with LMSs, a statistically significant difference was found. In this study, all ALM cases behaved in a benign fashion with no evidence of recurrence. Conversely, Chen and Yang [5] found a significant overlapping in p16 staining when comparing LMS with ALM, as up to 60% of ALM retained elevated p16 protein in tumor cells. O'Neill et al. [3] found that 5 of 11 ALMs exhibited staining of >50% of the tumor cells with p16. However, in the studies by Chen and Yang [5] and O'Neill et al. [3], the authors did not give the follow-up information of ALM cases.

Unlike p16, p21 targets a wide variety of CDK/cyclin complexes. p21 binds and inhibits CDK activity, in particular CDK2. In addition, p21 binds to the proliferating cell nuclear antigen (PCNA) to inhibit DNA replication. p21 protein expression seems to be a sign of intact cell cycle regulation. Abnormal expression of p21 has been observed in a variety of human tumors [22–25]. The diagnostic role of p21 immunohistochemistry in the field of gynecologic pathology is limited than that of p16. El-Ghobashy et al. [26] found a significant increase in p21 expression from normal cervix through endometriosis/tubo-endometrioid metaplasia and cervical glandular

intraepithelial neoplasia/adenocarcinoma in situ to invasive carcinoma. In contrast, Huang et al. [27] observed a trend of reduced p21 expression from normal cervical epithelium, low-grade squamous intraepithelial lesion and high-grade squamous intraepithelial lesion to microinvasive and invasive carcinoma. In the literature, there are few data regarding p21 expression in uterine smooth muscle tumors. In a series of Palazzo et al. [10], normal myometrial smooth muscle cells and 50% of LMs were negative for p21, whereas all LMSs showed focal or diffuse expression. In another series consisting of 36 uterine LMSs and 19 uterine LMs, p21 positivity (moderate or strong immunoreactivity) was found to be restricted to LMSs (15 of 36 cases), whereas all LMs were negative (negative or weak immunoreactivity) [11]. These results suggested that the p21 protein is an important element in LMS development. In our study, p21 protein was expressed in all cases of LMS and the percentage of staining was more than 10% in all except one case. Among the cases of LM, ALM and STUMP, only one case for each was positive for p21 in more than 10% of the tumor cells. There were statistically significant differences in the staining percentage and intensity with p21 between LMS and the other smooth muscle tumors (LM, ALM and STUMP). No statistically significant difference was observed between LM, ALM and STUMP. To the best of our knowledge, the present study is the first evaluating the role of P21 in STUMP cases. But due to a small number of cases, it is difficult to draw the conclusion for the role of p21 in STUMP.

Factors affecting prognosis in patients with LMS have not been consistent in different series. However, there is almost general agreement that tumor stage is the most significant predictor for overall survival [9, 11]. In our study, tumor stage was the only independent significant prognostic factor for overall survival in LMSs. We found that neither p16 nor p21 correlated with disease-free or overall survival. Our data are in agreement with other studies [2, 11].

In conclusion, accurate diagnosis of uterine smooth muscle tumors is crucial for clinical management of patients. Especially, unpredictable clinical behavior of STUMP makes clinical management of these patients problematic. Hence, it would be very useful to have additional markers which could help to distinguish these tumors. In the present study, we found statistically significant higher levels of p16 and p21 in LMSs compared to other groups. Our results suggest that these markers may be useful in the differential diagnosis of uterine smooth muscle tumors. Future studies based on larger case numbers are needed to confirm this suggestion.

Conflict of interest The authors declare that they have no conflict of interest.

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