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The expression of Ki-67, p53, estrogen and progesterone receptors affecting survival in uterine leiomyosarcomas: A clinicopathologic study

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Abstract

Objectives. To evaluate the level of expression of estrogen receptor (ER), progesterone receptor (PR), p53 and Ki-67 in patients with leiomyosarcoma and to investigate the effect of these and to identify the clinical parameters on prognosis.

Materials and methods. Twenty-four patients operated for LMS of uterine origin between 1994 and 2003 at Istanbul Medical School, Department of Obstetrics and Gynecology and Division of Gynecologic Oncology constituted our study group. The data of all patients were updated via mail or phone. The effects of stage, grade, chemotherapy, radiotherapy, number of mitoses, presence of necrosis, Ki-67 and p53 expression, presence of estrogen and progesterone receptors on survival were evaluated.

Results. The mean follow-up period of patients is 30.42 ± 25.15 months. The mean overall survival for all LMS patients was estimated to be 48.4 ± 10.38 months. The cumulative survival ratio in the 33rd month was 33.08. Age, menopausal status, history of prior radiotherapy, number of mitoses had no statistically significant effect on overall survival in our study although stage had a significant effect. Finding of greater than 10% steroid receptor expression has a positive effect on survival ([ER $P = 0.019$; log rank = 5.49] and [PR $P = 0.023$; log rank = 5.14]). The median value of Ki-67 was calculated to be 30. There was a survival advantage in patients with Ki-67 expression ($P = 0.034$; log rank = 4.49) below the median value. p53 levels had no significant effect on survival ($P = 0.336$; log rank = 0.92).

Conclusion. Surgical staging is an important prognostic factor in LMS patients, while number of mitoses and grade of the tumor also seem to affect prognosis. Contrary to the current literature, our findings suggest that estrogen and progesterone receptor positivity greater than 10% may be associated with a better prognosis.

Keywords: Leiomyosarcoma; Ki-67; p53; Estrogen receptor; Progesterone receptor

Introduction

Leiomyosarcoma (LMS) and endometrial stromal sarcoma (ESS) are rare malignant smooth muscle tumors of the uterus [1,2]. In spite of their rarity, they are hard to manage clinically due to their unpredictable recurrence rate and metastasizing capacity. Many clinicopathologic parameters affecting prognosis are investigated and among them most have controversial significance [3].

Tumor stage, histological grade, tumor size, menopausal status, age, mitotic index, tumor necrosis, and DNA ploidy are traditional prognostic factors which are previously studied. During the last decade, a number of researches have been done to assess estrogen receptors (ER), progesterone receptors (PR), and p53 and Ki-67 expression as prognostic factors in LMS [3–5].

Factors affecting transformation of uterine smooth muscle cells to LMS is not known, though ovarian steroids may have effect on this process. Many research papers have investigated the progesterone and estrogen receptor status in LMS: Zhai et al. [6] have found ER and PR expression in

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57% of LMS cases; Bodner et al. [4] have found 57% and 43% positivity for ER and PR, respectively. The authors have concluded that both of the parameters do not have any effect on overall and disease-free survival.

p53 which is a nuclear phosphoprotein, is involved in the control of cellular apoptotic pathway and loss of p53 function has been implicated in the pathogenesis of many human tumors [2]. Over-expression and mutation of the tumor suppressor p53 in LMS have been reported recently. Blom have proposed that p53 over-expression has a prognostic significance in terms of disease-free survival [2].

The cell proliferation associated antigen Ki-67 is found in all phases of the cell cycle except for phase-Go, so that the tumor growth fraction in some form may be quantified by measuring it. Although this parameter has been investigated previously, the only study investigating its association with prognosis in LMS has recently been published [5]. This study concluded that increased expression of Ki-67 have been associated with worse prognosis.

The aim of our study is to evaluate the level of expression of ER, PR, p53 and Ki-67 in patients with LMS and to investigate the effect of these parameters on prognosis.

Materials and methods

Subjects

Twenty-four patients operated for LMS of uterine origin between 1994 and 2003 at Istanbul Medical School, Department of Obstetrics and Gynecology and Division of Gynecologic Oncology constituted our study group. The patients were analyzed retrospectively. The surgical and medical data were retrieved from the archives of Division of Gynecologic Oncology, while macro- and microscopic data were gathered from the Department of Pathology of Istanbul Medical School.

Date of diagnosis, age, menopausal status, symptoms at diagnosis, date of operation, situs during the operation, histological type of the tumor, grade, status of adjuvant chemo- or radiotherapy, presence of recurrent disease and survival were recorded. All patients were staged according to the FIGO staging system of endometrial cancer.

All patients were contacted via mail or phone and had their data updated. If the patient was deceased, the reason and the date of death were recorded.

The morphological features were reassessed by reviewing all the archive slides and then a representative parafin block of each case was selected for the immunohistochemical examination.

Immunohistochemical procedure

Formalin-fixed and parafin embedded tissue was used for immunohistochemical procedure. Four-micron-thick sections were taken from each parafin block on coated (Zymed HistoGrip 00560472) glass slides. Following deparaffination and rehydratation, the slides were incubated with 3% hydrogen peroxide in methanol for 20 min to block endogen peroxidase activity, then boiled in citrate buffer solution (pH 6) using a domestic pressure cooker for antigen unmasking. After washing with phophate buffer solution (PBS), the slides were incubated with normal serum (Lab Vision Cor, large volume Ultra V Block, Fremont, CA) for 10 min to prevent nonspecific bindings. After washing with PBS, then the slides were incubated with monoclonal antibodies against Ki-67 (Neomarkers, Fremont, CA, MB 67, 1: 100 dilution, 30 min), estrogen receptor (Neomarkers, Fremont, CA, 1D5+6F11, 1:100 dilution, 60 min), progesterone receptor (Neomarkers, Fremont, CA, hPRa 2+hPRa 3, 1:100 dilution, 60 min) and p53 (Neomarkers, Fremont, CA, DO-7, 1:100 dilution, 30 min). Then, the slides were washed with PBS again and incubated with secondary antibody (Lab Vision Cor, biotinylated goat anti-mouse, Fremont, CA, dilution 1:25) for 25 min and with streptavidin–horse radish peroxidase solution (Lab Vision Cor, large volume streptavidin peroxidase, Fremont, CA) for 25 min. Aminoethyl carbasole was used as chromagen and Mayer's hematoxylin as counterstain. A tonsil tissue block for Ki-67 and a breast carcinoma block for estrogen receptor, progesterone receptor and p53 were used as positive controls. The same procedure was done in order to omit the incubation with primary antibodies for negative controls. All the procedure was done at room temperature.

Microscopic analysis

All the archive glass slides were reviewed for microscopic analysis. To reach a diagnosis of uterine leiomyosarcoma, nuclear atypia, mitotic rate and coagulative tumor cell necrosis were the most important features which were especially looked for each cases, in addition to other features, including status of tumor borders, epitheloid cells and myxoid background (Table 1). Grading was done according to conventional differentiation degree of the tumor for each case without taking the mitotic count and amount of necrosis into account. To determine the final mitosis score, first we screened all the slides to find the most active areas using low power magnifications ($\times$10 and $\times$20) and then we counted 10 consecutive high power fields ($\times$40). In immunohistochemical analysis for p53 and Ki-67, prior to counting, we screened all the slides using low power magnification to find the hot spots (showing high amount of neoplastic cells with positive immunostaining to find the percentage of the positive staining). Then, we counted at least a thousand neoplastic cells in the hot spots to find the ratio of positively staining cells, using the oil objective ($\times$100). We also counted all positive staining cells in an area of tumor, measuring 2 mm^2 (11 consecutive $\times$40 areas) to find the amount of positive staining cells in 1 mm^2. For estrogen and progesterone receptor, we screened all the

Table 1
Age, grade, necrosis, stage, follow-up, status and expression of p53, Ki-67, ER, PR in leiomyosarcomas

	Age	Grade	p53[a]	p53 mm^{2b}	Ki-67[a]	Ki-67mm^{2b}	ER[c]	PR[c]	ER (%)[d]	PR (%)[d]	Necrosis	Stage	Follow-up (months)	Status
1	66	3	10.95	70	5.12	73	–	–	0	0	+	1	109	A
2	71	3	0	0	8.95	294	–	–	0	0	–	3	24	D
3	44	3	9.05	97	51.47	1784	–	–	0	0	+	4	9	D
4	52	2	0	0	4	960	–	+	0	20	–	3	33	D
5	63	2	5.14	36	25.16	452	–	–	0	0	+	2	26	D
6	70	2	0	0	13.62	414	–	–	0	0	+	4	8	D
7	50	2	26.57	598	34.02	591	+	+++	60	90	–	1	22	A
8	38	1	24.10	293	25.91	868	–	–	0	0	+	3	29	D
9	39	1	0	0	14.90	180	+	+++	30	90	–	1	58	A
10	28	1	1	1	2.56	10	+	++	40	80		1	67	A
11	60	1	3.74	68	8.09	148	+	+++	30	80	+	2	39	A
12	44	2	0	0	38.37	1323	+	+	5	1	+	3	12	D
13	77	1	46.54	973	57.28	1199	–	–	0	0	+	3	31	D
14	66	2	8.04	1672	41.53	891	–	–	0	0	+	4	17	D
15	57	3	90	1837	55.72	1057	–	–	0	0	+	3	9	D
16	49	2	1	2	59.61	1807	–	+	0	5	–	1	27	A
17	76	3	18	921	47.3	760	–	–	0	0	+	2	14	D
18	49	1	12	121	30	157	–	–	0	0	+	1	43	A
19	63	3	10.9	57	49	694	–	–	0	0	–	4	10	D

A: Alive; D: Dead.
[a] The percentage of the positive staining for p53 and Ki-67.
[b] Stained cells in 1 mm^2 for p53 and Ki-67.
[c] The immunostaining intensity of estrogen receptor (ER) and progesterone receptor was classified into three grades: weak, moderate and strong.
[d] The percentage of positive immunostaining of estrogen and progesterone receptors.

section using both low and high power magnifications to find the approximate percentage of positive immunostaining and also staining intensity. A positive staining was considered if there was a diffuse nuclear staining for all of the antibodies. All the microscopic examination was done using an Olympus BH2 light microscope (diameter at high power field = approximately 0.48 mm).

Classification of data and statistical analysis

The follow-up period and the age of the patients with LMS are expressed in mean, minimum and maximum values. The effects of stage, grade, chemotherapy, radiotherapy, number of mitoses, presence of necrosis, Ki-67, p53, and presence of estrogen and progesterone receptors on survival were evaluated.

Since there were insufficient data about the immunoreactivity of Ki-67 in uterine sarcomas, median value was used as the cut-off value during our statistical analysis. Survival of the patients that were above and below this median value was compared. Similarly, the threshold value for p53 in the literature is in discord. Some authors have defined this value as 5% or 10% stained area during immunohistochemical procedures, while others have grouped the patients due to the mere presence or absence of the stain. In our study, 5% stained area was used as the cut-off value. For estrogen and progesterone receptor levels, 10% cut-off level that was used by our pathologists was accepted.

Fisher's Exact Test was used for comparing stage and steroid receptor positivity. Survival in months was calculated as the period from the operation to the patient's last follow-up. Kaplan–Meier method was used to calculate the mean survival and to evaluate factors affecting overall survival (Table 2). Using the log-rank procedure, the resultant curves were compared. Statistical significance was accepted for values less than 0.05 ($P < 0.05$).

Results

Retrospective analysis of data of Istanbul University, Istanbul Medical School, Department of Obstetrics and Gynecology, Division of Gynecologic Oncology between 1992 and 2002 revealed 1465 patients with the diagnosis of malignant genital tumor. Out of these, 28.3% (415/1465) were diagnosed to have malignant uterine tumor. Fifty-three of these cases were uterine sarcomas. Uterine sarcomas comprised 3.6% and 12.7% of all malignant genital and uterine tumors, respectively.

Out of these 53 cases, 11 were mixed mullerian tumor (MMT) and 5 were unclassified sarcomatous uterine tumors. Twenty-four patients had LMS while 13 had ESS. One of the patients with LMS could not be contacted. Due to technical problems, the paraffin blocks were unavailable for immunohistochemical analysis for 4 patients. The data of a total of 19 patients with LMS were examined.

The mean age of 19 patients with LMS was 55.89 ± 13.76 years. The distribution of the age of patients is depicted in Table 4. The mean follow-up period of patients was 30.42 ± 25.15 months. The mean overall survival for all LMS patients was estimated to be 48.4 ± 10.38 months. The

Table 2
Survival analysis of the patients (Kaplan–Meier method) (St.Er. = standard error – 95% CI = 95% confidence interval)

Survival parameters for leiomyosarcomas	*n*	Mean S.T. (month) ± St.Er.	95% CI	Log rank	*P*
Overall survival for leiomyosarcomas	19	48.4 ± 10.38	28.05–68.75		
Age <60	10	40.10 ± 8.43	23.57–56.63	0.86	0.353
Age >60	9	38.67 ± 12.75	13.68–63.66		
Premenopausal	9	43.33 ± 8.87	25.96–60.71	1.03	0.31
Postmenopausal	10	37.60 ± 11.6	14.86–60.34		
Stage I + II	9	87.90 ± 13.08	62.27–113.54	11.91	0.0006
Stage III + IV	10	17.30 ± 2.97	11.49–23.11		
Grade I	8	78.63 ± 13.92	51.34–105.91	9.53	0.002
Grade II + III	11	16.52 ± 2.22	12.16–20.87		
Chemotherapy and/or radiotherapy (+)	8	33.88 ± 14.37	5.72–62.03	1.89	0.168
Chemotherapy and/or radiotherapy (−)	11	40.89 ± 7.27	26.64–55.14		
Mitotic index (<10)	7	80.86 ± 16.82	47.89–113.83	3.55	0.059
Mitotic index (>10)	12	22.56 ± 3.24	16.20–28.91		
Necrosis (+)	13	44.77 ± 12.01	21.24–68.30	0.54	0.463
Necrosis (−)	6	35.10 ± 8.16	19.11–51.10		
Ki-67 < median of the value	10	65.60 ± 13.85	38.46–92.74	4.49	0.034
Ki-67 > median of the value	9	18.22 ± 3.36	11.63–24.82		
Ki-67 mm^2 < median of the value	9	76.44 ± 15.09	46.87–106.02	6.44	0.011
Ki-67 mm^2 > median of the value	10	18.07 ± 3.13	12.57–24.83		
p53 (<5% stained area)	9	40.28 ± 8.11	24.39–56.17	0.92	0.336
p53 (>5% stained area)	10	41.10 ± 13.79	14.07–68.13		
p53 mm^2 (<5% stained area)	7	38.10 ± 9.47	19.54–56.65	0.25	0.616
p53 mm^2 (>5% stained area)	12	44.98 ± 12.77	19.96–70.01		
Estrogen receptor (<10%)	15	33.43 ± 9.42	14.97–51.89	5.49	0.019
Estrogen receptor (>10%)	4	All the patients were alive			
Progesterone receptor (<10%)	14	34.36 ± 10.31	14.14–54.57	5.14	0.023
Progesterone receptor (>10%)	5	58.50 ± 7.36	44.07–72.93		

cumulative survival ratio in the 33rd month was 33.08. During the study period, 12 patients were lost and 9 patients had recurrences (47%). The recurrences were 4 in the lung, 2 in the abdominal cavity, 1 in vagina, 1 in lung and abdominal cavity (GIS), and 1 in vagina and abdominal cavity. In two deceased patients, no information about possible recurrences was gathered. Analysis of factors affecting the recurrence rate was omitted due to lack of information. Six of the patients were operated twice (31.5%) while 13 had only one operation.

The two most important parameters affecting survival in LMS patients were stage and tumoral grade. Age, menopausal status, history of prior radiotherapy, and number of mitoses had no statistically significant effect on the overall survival in our study. Although the number of mitoses lacked statistical significance, the *P* value was very close to being statistically significant ($P = 0.059$).

Distribution of patients with positive ER and PR is detailed in Table 4. Although advanced stage LMS tumors have low estrogen and progesterone receptor expression (Table 3), no statistically significant relevance was found between stage and percentage of positive immunostaining of steroid receptor (ER vs. stage $P = 0.135$; PR vs. stage $P = 0.129$). Finding of greater than 10% steroid receptor expression has a positive effect on survival (Fig. 1). Only receptor positivity was used during calculation of survival analysis likewise in other relevant publications (Table 3); so applying the same method, the correlation between steroid receptor expression and prognosis was close to being significant ($P = 0.065$; log rank = 3.39).

The median value of Ki-67 was calculated to be 30 in our study. After comparison of cases which were below and above this cut-off value, there was a survival advantage in patients with low Ki-67 expression (Fig. 2). Levels of p53 had no significant effect on survival.

Discussion

Uterine sarcomas are rare, aggressive tumors with high metastatic capacity and recurrence rates even if diagnosed early. The unknown factors affecting to uterine sarcomas and the adjuvant therapies, which do not affect survival are the two main problems while dealing with uterine sarcomas [1]. Surgical staging and tumor grade are the only parameters agreed upon to affect the prognosis [1,2]. Likewise, surgical staging is an important prognostic factor

Table 3
Patients distributed according to the stage

	n (%)	ER (>10%) (%)	PR (>10%) (%)
Stage I	6 (31.5)	3 (50)	4 (66)
Stage II	3 (16)	1 (33)	1 (33)
Stage III	6 (31.5)	1 (17)	2 (33)
Stage IV	4 (13)	0	0
Total	19	5 (26)	7 (37)

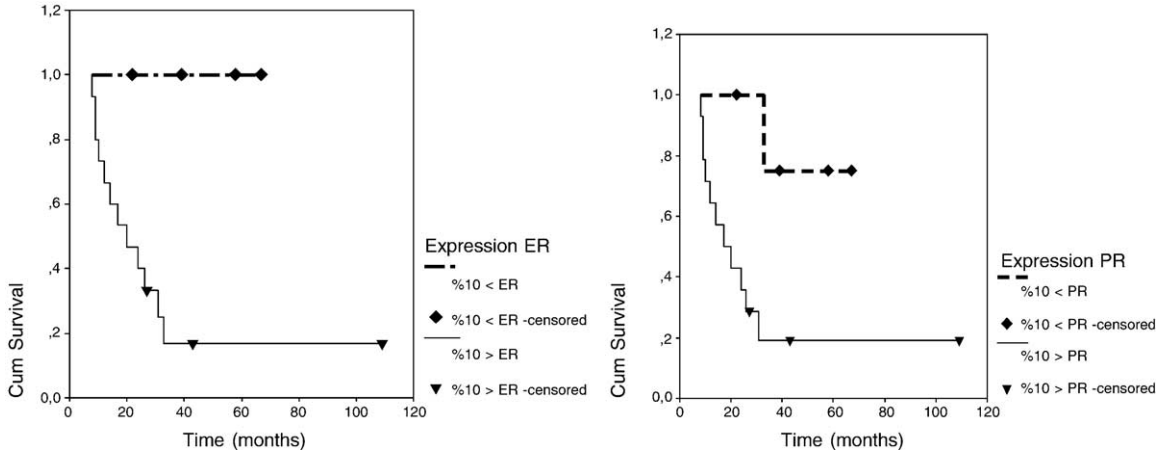

Fig. 1. Greater than 10% of estrogen and progesterone receptor expression has a positive effect on survival (ER $P = 0.019$ and PR $P = 0.023$). ER: Estrogen receptor, PR: Progesterone receptor.

in our study, while number of mitoses and grade of the tumor also seem to affect prognosis.

Since Sutton et al. [7] and Soper's et al. [8] investigations on the relationship between the steroid receptors and survival along with other various clinical parameters in uterine sarcomas, the topic has been a subject of interest of many authors. Although the relationship between the steroid receptors and tumor growth in breast and endometrial cancer models is explained, it still remains unsolved in uterine sarcomas. The resolution of this issue would serve to understand the dynamics of sarcomatous growth; hence the prognosis and the possible use of hormonal therapy in the treatment of these tumors. Published studies are few in number, cases are rare (Table 4) and their results are controversial. The rarity of these tumors may not be the only explanation, the analysis of different types of sarcomas under one title may have also contributed to this problem: Although LMS, ESS and MMT are analyzed together, both histologically and biologically each of them is a different type of sarcoma. The study of Livi and colleagues [1] has demonstrated that all three are very different from each other. Especially, MMT, which is considered to be a carcinoma with sarcomatoid microscopic features, differs from the others by containing epithelial components and its prognosis is far worse. Apart from classical prognostic factors like mitotic index and necrosis, our study tries to evaluate the effect of new parameters like sex steroids, Ki-67 and p53 on prognosis. If all histopathological types of sarcomas were evaluated together without further subdividing them, the quality of data would certainly be affected adversely.

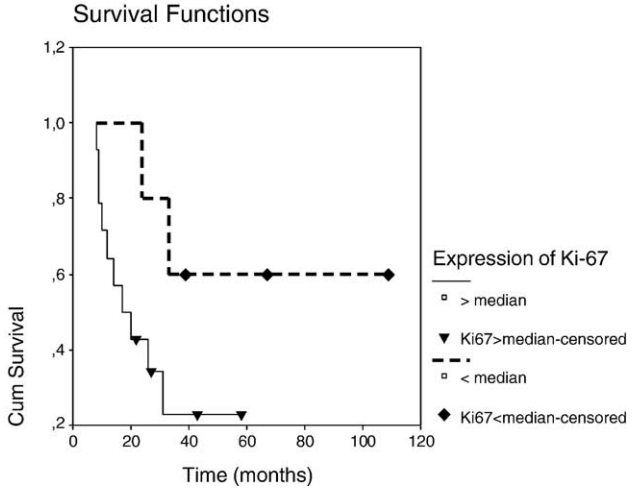

Fig. 2. There was a survival advantage in patients with low Ki-67 expression ($P = 0.034$).

Limited studies evaluating the relationship between estrogen–progesterone receptors positivity and stage or grade have shown that these parameters are not related with each other. Others proposed that survival is also not related to ER and PR positivity. In the study of Kitaoka et al. [9] from 31 cases, of which 22 are LMS, it proposed that ER and PR positivity and survival were not correlated; while in the study of Sutton et al. [7] of 39 cases, ER and PR positivity was found to be significantly higher in patients with LMS living longer than 1 year after diagnosis.

Contrary to the current literature, our findings suggest that estrogen and progesterone receptor positivity greater than 10% was associated with a better prognosis. In addition, ER and PR positivity in our data was found to be less than the current literature (Table 4). Clinical staging may account for this difference. Although Bodner's study [4], which is supported by Sutton's findings, stated that clinical stage and ER–PR positivity were not correlated, there is only one case of stage IV disease and that tumor is ER and PR negative. Sutton's data contain only 7 cases of LMS and Kitaoka's data contain only 4 cases of stage IV LMS out of 22 cases. All of these tumors are PR and only one is weakly ER positive. In our data, 13% of the cases are stage IV disease and likewise all are ER–PR negative. Even though there was no statistically significant relationship

Table 4
Distribution of LMS patients in literature according to the stage, ER and PR positivity

Authors	Year	*n*	Stage I (%)	Stage II (%)	Stage III (%)	Stage IV (%)	ER (+) (%)	PR (+) (%)
Kitaoka Y.	2003	22 LMS	14 (64)	0	4 (18)	4 (18)	8/22 (36)	8/22 (36)
Bodner K.	2003	21 LMS	13 (61)	3 (14)	4 (19)	1 (5)	12/21 (57)	9/21 (43)
Zhai Y.	1999	14 LMS	10 (72)	1 (7)	2 (14)	1 (7)	5/14 (36)	8/14 (57)
Wade K.	1990	16 LMS	6 (37.5)	1 (6.2)	4 (25)	5 (31.2)	9/16 (60)	9/16 (60)
Sutton G.P.	1986	7 LMS	3 (43)	1 (14)	1 (14)	2 (29)	*	*
Akhan S.E.		19 LMS	6 (31.5)	3 (16)	6 (31.5)	4 (13)	5/19 (26)	7/19 (37)

*Details unavailable in paper.

between stage and steroid receptor positivity in our data, limited number of cases may have affected statistical significance.

The main problem in all of these studies, including ours, is that the number of cases is limited to make an ideal statistical analysis. Estrogen and progesterone receptor positivity had a beneficial effect on prognosis. Our analysis is based on 10% cut-off value used by our university's department of pathology while in the literature only the positive results were taken into account. When our data are analyzed according to estrogen and progesterone receptor positivity, the results were close to being statistically significant. Due to the limited number of cases, it is very hard to judge scientifically whether a true relationship exists between these parameters or not.

From the 1990s, especially by pathologists, expression of Ki-67 and p53 has been used to differentiate among uterine smooth muscle tumors of uncertain malignant potential (STUMP), atypical leiomyoma, and LMS. It is also used to evaluate the malignant potential of uterine smooth muscle tumors. Zhai et al. [6] have shown that Ki-67 expression is significantly higher in LMS than STUMP's and atypical leiomyoma confirming Amada's results [10]. Since the cut-off value for Ki-67 was not defined in the literature, we have taken the median value as cut-off and our analysis has shown that increased Ki-67 expression adversely affects prognosis. This result is similar to the findings of two studies of Mayerhofer in 2004 [5,11]: increased Ki-67 expression is correlated with rapid growth of the malignant tumor and worse prognosis. Our study supports the proposal that increased Ki-67 expression is an adverse prognostic factor when present.

As previously mentioned, p53 expression in LMS is a topic of interest since the 1990s. Amada et al. [10], Niemann et al. [12], and Sprogoe-Jacobsena and Holund [13] have found 33% (8/24), 47% (16/34) and 71% (5/7) positivity of p53 expression in LMS patients, respectively. Layfield et al. [3] also in their study concluded that a relationship between p53 expression and prognosis exists. However, Blom et al. [2] have found 31% expression and suggested that p53 was not related to prognosis but related to recurrences. Our data suggested that there was no relationship between p53 expression and prognosis although in 53% of the cases the results were positive. Our results are in accordance with Blom's study.

In conclusion, estrogen and progesterone receptor positivity more than 10% and low expression of Ki-67 antigen expression may be parameters, which positively affect survival. As we emphasized before, the most significant prognostic factor was "the stage." Multivariate analysis should have been performed in order to evaluate other parameters objectively, which we think that are effective on survival and we could not perform this analysis because of inadequate number of cases. More prospective studies performed on only one subtype of sarcoma with a large number of patients are needed to clarify the issue.

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