Leiomyosarcoma Versus Bizarre and Cellular Leiomyomas of the Uterus: A Comparative Study Based on the MIB-1 and Proliferating Cell Nuclear Antigen Indices, p53 Expression, DNA Flow Cytometry, and Muscle Specific Actins

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Leiomyosarcoma Versus Bizarre and Cellular Leiomyomas of the Uterus: A Comparative Study Based on the MIB-1 and Proliferating Cell Nuclear Antigen Indices, p53 Expression, DNA Flow Cytometry, and Muscle Specific Actins

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Summary: There is still controversy over the criteria for malignancy of smooth muscle tumors (SMTs) of the uterus. We examined 51 cellular SMTs using immunohistochemistry for MIB-1, proliferating cell nuclear antigen (PCNA), p53, HHF35, α-smooth muscle actin (SMA), and flow cytometry. Morphologically, the 51 cases were classified into 24 leiomyosarcomas (LMS), two uncertain malignant potential, four bizarre leiomyomas, and 21 cellular leiomyomas. The mean values of the MIB-1 and PCNA indices showed significant differences between LMS and benign SMTs. p53 cells were positive in eight of 24 leiomyosarcomas, and 12 of 22 were aneuploid. HHF35 and α-SMA showed a diffuse positivity in almost all the benign SMTs. In contrast, 10 of the 24 LMS were either focally positive or negative for SMA. Using a logistic regression model, at cut-off points of 3.6 on the MIB-1 index and 15.6 on the PCNA index, the LMS and the benign SMTs were classified with an overall accuracy of 92% and 82%, respectively. Moreover, by combining the MIB-1 index and α-SMA positivity, the cut-off point could be established at 0.492 on the probability scale with the highest overall accuracy of 96%. Regarding the prognosis of LMS, p53 positivity was correlated with survival (p = 0.0357). A combination of the MIB-1 index and α-SMA was helpful in distinguishing between LMS and benign SMT. Moreover, p53 positivity was considered to be a good marker for predicting the prognosis of LMS.

Key Words: Leiomyosarcoma—Ki-67—Proliferating cell nuclear antigen—p53—Flow cytometry—Muscle-specific actins.
LEIOMYSARCOMA OF THE UTERUS

assess the correlation between their biological characteristics and histological findings using both immunohistochemical techniques and flow cytometry.

MATERIALS AND METHODS

Fifty-one cases of uterine cellular SMTs were selected from our files of gynecological neoplasms in the Second Department of Pathology, Kyushu University, from 1977 to 1992. Cases initially labeled as leiomyosarcoma (LMS), cellular leiomyoma (CL), bizarre leiomyoma (BL), and uncertain malignant potential category (UMP) were studied. The clinical information, including follow-up data, was obtained from both the patients’ physicians and medical records. Follow-up data for all patients except for one with LMS were available. Hematoxylin and eosin preparations in the proper number for each tumor size were reviewed.

Histologic assessment

The following histologic features were semiquantitatively evaluated. The nuclear atypia of each case was determined by dividing the range of nuclear features from mild to severe. Mildly atypical nuclei possessed regular borders and an even distribution of chromatin, similar to that of benign CL. Severely atypical nuclei were large and pleomorphic, with an irregular nuclear shape, clumped chromatin, and prominent nucleoli. Moderately atypical nuclei ranged between mild and severe. Mitoses were counted and expressed as the number of mitotic figures/10 high-power fields (hpf). Count was made on a binocular Olympus BH-2 microscope using 40× S-Plan Apo objective and 10×/20L wide-field eyepieces while selecting the most active areas where mitoses were the most frequent. The presence of coagulative tumor necrosis was examined in all the given specimens of each case.

Based on the above histological factors, including the criteria of hypercellularity, cellular atypia, mitotic count, and the presence of tumor necrosis based on previous reports (1-8), the 51 cases were diagnosed into LMS, UMP, BL, and CL. LMS were further classified into four subgroups: (a) moderate to severe nuclear atypia and 10 and more mitotic figures/10 hpf; (b) moderate to severe nuclear atypia and five to nine mitotic figures/10 hpf; (c) mild nuclear atypia and 10 or more mitotic figures/10 hpf; (d) mild nuclear atypia and five to nine mitotic figures/10 hpf. LMS and benign SMT (BL and CL) were used for the immunohistochemical and flow cytometric studies.

Immunohistochemistry

All sections from the formalin-fixed and paraffin-embedded specimens were studied immunohistochemically using avidin-biotin complex methodology (17). The primary antibodies and their dilutions are summarized in Table 1. All the sections were deparaffinized with xylene and dehydrated in a graded series of alcohol. The sections for Ki-67 (MIB-1) were pretreated with boiling in citrate buffer, pH 6.0, for 30 min. All the sections were incubated in 0.3% hydrogen peroxidase in methanol for 30 min and then were exposed to the primary antibodies at given dilutions by phosphate-buffered saline at room temperature for 90 min. A subsequent reaction was made by using an avidin-biotin-peroxidase kit (Nichirei, Tokyo, Japan). The sections were stained with freshly prepared diaminobenzidine (DAB) solution and then were counterstained with methyl green or hematoxylin.

Assessment of immunohistochemical staining

MIB-1, PCNA, and p53 staining was confined to the nuclei. The MIB-1 and PCNA indices, as the extent of MIB-1 and PCNA positivity in each case, respectively, were evaluated by determining the percentage of positive nuclei counted at high power and more than three fields that were chosen at random. A p53-positive case was defined as a case with positive cells, whereas a case that expressed sporadic positive cells was excluded from the positive category. HHF35 and α-SMA staining were categorized into four grades expressed as negative (−), focally positive (1+; <50% of cells had a positive reaction), intermediately positive (2+; 1+ to 3+), and diffusely positive (3+; almost all the tumor cells had a positive reaction).

Flow cytometry

The flow cytometric determination of tumor cell DNA content was performed on paraffin-embedded sections using an earlier published method (18, 19).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki-67 (MIB-1) monoclonal</td>
<td>Immunotech S.A.</td>
<td>1:100</td>
</tr>
<tr>
<td>PCNA (PC10) monoclonal</td>
<td>DAKO</td>
<td>1:100</td>
</tr>
<tr>
<td>p53 (CM1) polyclonal</td>
<td>Novocastra</td>
<td>1:500</td>
</tr>
<tr>
<td>HHF35 monoclonal</td>
<td>DAKO</td>
<td>1:100</td>
</tr>
<tr>
<td>α-SMA monoclonal</td>
<td>Sigma Chemical</td>
<td>1:5000</td>
</tr>
</tbody>
</table>

The cellular DNA content was measured on a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA) with a 488-nm argon ion laser. Histograms of 1 x 10⁶ cells were recorded and analyzed with CellFIT cell-cycle analysis software (Becton Dickinson). The first G0/G1 peak was assumed to be a diploid population, and DNA aneuploidy was defined by the presence of a well-defined second peak.

**Statistical methods**

A logistic regression model of the BMDP statistical Package Program (BMDP Statistical Software Inc., California) on the IBM system 4381 computer (Armonk, New York, NY) was used to calculate the probabilities of a malignant or benign category (20). From this analysis, the equations and optimal thresholds for classifying the cases into either a malignant or a benign category were calculated (21). The accuracy of classification was determined for each individual parameter (MIB-1 and PCNA indices), and subsequently for more than one variable (adding p53, HHF35, and α-SMA status) to further improve accuracy. The survival rates of the cases with LMS were calculated by the Kaplan-Meier method, and comparisons were made using the generalized Wilcoxon test. Other statistical differences were evaluated with the use of either the Student's t test or the χ² test.

**RESULTS**

**Histological features**

Based on the findings previously mentioned in Materials and Methods, the cellular SMTs were classified into 24 LMS, 10 UMPs, four BLs, and 21 CLs. In the 24 LMS, group A consisted of 13 cases; the highest mitotic count was 60 mitotic figures/10 hpf, the average 31.8 mitotic figures/tO hpf. Eight of the 13 cases showed severe nuclear atypia and tumor necrosis. Another tumor showed moderate atypia and no necrosis. Group B, which consisted of three cases, two of the three showed severe nuclear atypia and tumor necrosis. Another tumor showed moderate atypia and no necrosis. Group C consisted of five neoplasms with a range of 10-16 mitotic figures/10 hpf. Eight of the 13 cases in group C disclosed nuclear atypia and no necrosis. Group D included three tumors, all of which showed coagulative necrosis. Two neoplasms exhibiting two and three mitotic figures/10 hpf, moderate nuclear atypia, and no tumor cell necrosis were labeled as UMPs, according to the published criteria (1.2).

**TABLE 2. Histological findings of leiomyosarcomas and UMP tumors**

<table>
<thead>
<tr>
<th>Case</th>
<th>Age (yr)</th>
<th>Mitoses</th>
<th>Atypia</th>
<th>Nucleoli</th>
<th>Prognosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>63</td>
<td>60</td>
<td>Severe</td>
<td>5-9</td>
<td>DOD (19)</td>
</tr>
<tr>
<td>2</td>
<td>49</td>
<td>30</td>
<td>Severe</td>
<td>5-9</td>
<td>DOD (3)</td>
</tr>
<tr>
<td>3</td>
<td>62</td>
<td>30</td>
<td>Severe</td>
<td>5-9</td>
<td>DOD (48)</td>
</tr>
<tr>
<td>4</td>
<td>85</td>
<td>25</td>
<td>Severe</td>
<td>5-9</td>
<td>DOD (10)</td>
</tr>
<tr>
<td>5</td>
<td>60</td>
<td>22</td>
<td>Severe</td>
<td>5-9</td>
<td>DOD (17)</td>
</tr>
<tr>
<td>6</td>
<td>43</td>
<td>20</td>
<td>Severe</td>
<td>5-9</td>
<td>DOD (17)</td>
</tr>
<tr>
<td>7</td>
<td>54</td>
<td>20</td>
<td>Severe</td>
<td>5-9</td>
<td>DOD (3)</td>
</tr>
<tr>
<td>8</td>
<td>54</td>
<td>20</td>
<td>Severe</td>
<td>5-9</td>
<td>DOD (3)</td>
</tr>
<tr>
<td>9</td>
<td>47</td>
<td>15</td>
<td>Severe</td>
<td>5-9</td>
<td>NED (6)</td>
</tr>
<tr>
<td>10</td>
<td>48</td>
<td>15</td>
<td>Severe</td>
<td>5-9</td>
<td>NED (6)</td>
</tr>
<tr>
<td>11</td>
<td>43</td>
<td>15</td>
<td>Severe</td>
<td>5-9</td>
<td>NED (6)</td>
</tr>
<tr>
<td>12</td>
<td>47</td>
<td>15</td>
<td>Severe</td>
<td>5-9</td>
<td>NED (6)</td>
</tr>
<tr>
<td>13</td>
<td>43</td>
<td>15</td>
<td>Severe</td>
<td>5-9</td>
<td>NED (6)</td>
</tr>
<tr>
<td>14</td>
<td>32</td>
<td>15</td>
<td>Severe</td>
<td>5-9</td>
<td>NED (6)</td>
</tr>
<tr>
<td>15</td>
<td>32</td>
<td>15</td>
<td>Severe</td>
<td>5-9</td>
<td>NED (6)</td>
</tr>
<tr>
<td>16</td>
<td>32</td>
<td>15</td>
<td>Severe</td>
<td>5-9</td>
<td>NED (6)</td>
</tr>
<tr>
<td>17</td>
<td>32</td>
<td>15</td>
<td>Severe</td>
<td>5-9</td>
<td>NED (6)</td>
</tr>
<tr>
<td>18</td>
<td>32</td>
<td>15</td>
<td>Severe</td>
<td>5-9</td>
<td>NED (6)</td>
</tr>
<tr>
<td>19</td>
<td>32</td>
<td>15</td>
<td>Severe</td>
<td>5-9</td>
<td>NED (6)</td>
</tr>
<tr>
<td>20</td>
<td>32</td>
<td>15</td>
<td>Severe</td>
<td>5-9</td>
<td>NED (6)</td>
</tr>
<tr>
<td>21</td>
<td>32</td>
<td>15</td>
<td>Severe</td>
<td>5-9</td>
<td>NED (6)</td>
</tr>
<tr>
<td>22</td>
<td>32</td>
<td>15</td>
<td>Severe</td>
<td>5-9</td>
<td>NED (6)</td>
</tr>
<tr>
<td>23</td>
<td>32</td>
<td>15</td>
<td>Severe</td>
<td>5-9</td>
<td>NED (6)</td>
</tr>
<tr>
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<td>32</td>
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<td>Severe</td>
<td>5-9</td>
<td>NED (6)</td>
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<td>Severe</td>
<td>5-9</td>
<td>NED (6)</td>
</tr>
<tr>
<td>26</td>
<td>32</td>
<td>15</td>
<td>Severe</td>
<td>5-9</td>
<td>NED (6)</td>
</tr>
</tbody>
</table>

**Clinical features**

The age range of the patients with LMS was 38-63 years (mean 48.7). All patients with LMS were treated with a total hysterectomy as primary therapy. The initial laparotomy disclosed extrauterine lesions in two women of group A. Twelve women, including the two mentioned above, died of disease at 2-210 months (mean 34.4) after surgery and were from all of the four groups. One woman from group D is now being treated for a recurrent tumor. Both women with UMPs are alive without any sign of disease for 14 and 59 months after surgery, respectively.

**DNA ploidy pattern**

Analyzable DNA histograms were obtained from 44 of the tumors studied. The mean coefficient of variation was 5.3% (range 2.2-12.5). Twelve of the 22 LMS exhibited a DNA-aneuploid cell population, whereas all the benign SMTs showed a diploid DNA histogram. The DNA ploidy pattern of the LMS and the benign SMTs (BLs and CLs) significantly differed between themselves (p < 0.0001, Table 3).

**Muscle-specific actins**

HHF35 and α-SMA were seen almost diffusely (the value exhibited 2+ or 3+) in all the benign BLs and CLs except for two cases. In contrast, 10 of 24 LMS showed either focal or negative immunoreactivity for HHF35 or α-SMA (0 or 1+). The difference of immunostaining for α-SMA was statistically significant between the LMS and the benign SMTs (BLs and CLs) (p = 0.0004) (Table 3).

**Logistic regression model**

The probability of being an LMS or a benign SMT (BL or CL) was computed using a logistic regression equation with either the MIB-1 or PCNA index. To determine the accuracy of classification into LMS and benign SMT (BL or CL), the probability at different cut-off points was tested. For the MIB-1 index, at a cut-off point of 0.492 probability (MIB-1 index 3.6), 87% (21 of 24) of the LMS and 96% (24 of 25) of the benign SMTs, overall 92%, were correctly classified. Regarding the PCNA index, at a cut-off point of probability of 0.525 (PCNA index 15.6), 75% (18 of 24) of LMS and 84% (21 of 25) of the benign SMTs, overall 82%, were also correctly classified (Table 4). When all possible combinations of more than one parameter (MIB-1 and PCNA indices, p53 and muscle-specific actins) were considered, the highest
over all accuracy was achieved by combining the MIB-1 index and α-SMA. A cut-off probability of 0.492, 96% (23 of 24) of the LMS and 96% (24 of 25) of the benign SMT, 96% overall, were correctly classified (Table 4).

Survival

In 23 LMS, there were no differences between the survival and mitotic count (groups A and C vs. B and D) or nuclear atypia (groups A and B vs. C and D). Regarding the immunohistochemical and flow cytometric factors, LMS had a tendency to correlate with survival and the p53 positivity with statistical significance (p = 0.0357, Fig. 6). However, there was no significant correlation between the other factor (MIB-1, PCNA, muscle-specific actins, or DNA ploidy pattern) and the prognosis.

DISCUSSION

Several investigators have reported on the diagnostic criteria for uterine LMS, as shown by hypercellularity, cellular atypia, and numerous mitotic figures (1-8). The most reliable factor of the diagnosis is believed to be the mitotic count (13,6). However, there is a small controversial group of the uterine SMTs with cellular atypia and only a slight amount of mitotic activity (<10 mitotic figures/10 hpf), in which malignancy is difficult to determine.

In our experience, six patients who were diagnosed with LMS showed <10 mitotic figures/10 hpf, and four of them died of the disease at 12-210 months after surgery. In the groups with mild nuclear atypia, two patients died from the disease while histologically showing only 13 and 8 mitotic figures/10 hpf, and one patient with six mitotic figures/10 hpf has a history of a recurrence. On the other hand, no patients with fewer than five mitotic figures/10 hpf had either recurrence or a fatal course. Thus, a definition to distinguish between benign and malignant SMTs might be set at five mitotic figures/10 hpf if one does not need to bother about the problem of accuracy in counting mitosis as discussed in the literature (22-25).

MIB-1 (345 and 395 kDa double band in Western blot analysis) is a new antibody that reacts with the Ki-67 nuclear antigen in paraffin-embedded sections, is associated with cell proliferation, and is found throughout the cell cycle of G1, S, G2, and M phases (26,27). Several studies of MIB-1 on neoplasms have been reported (28). PCNA is a 36-kDa acidic nonhistone nuclear protein that functions as an auxiliary protein for DNA polymerase β and is an absolute requirement for DNA synthesis (29). In mesenchymal neoplasms, a large series of PCNA immunostaining has been performed on a few studies of hemangiopericytoma by Yu et al. (12) and synovial sarcoma by Oda et al. (11). No series of MIB-1 and PCNA immunostaining have been previously performed on the uterine SMTs. We found a significant difference (p < 0.0001) in the MIB-1 and PCNA indices between the LMS (39.2 and 35.2) and benign SMTs (BL and CL) (1.1 and 6.2). The mitotic count, the most reliable diagnostic morphological factor in previous studies (1,3-6), shows only the number of proliferating cells in the M phase. But the proliferating cells occupy several functional states besides mitosis. Ki-67 is found in proliferating cells of the G1, S, G2, and M phases (27), and PCNA is synthesized in the late G1 and S phases (29). Therefore, our results of the MIB-1 and PCNA indices make it clear that the number of proliferating cells is important to discriminate between LMS and benign SMTs. Indeed, at a cut-off point of 3.6 for the MIB-1 index and at 15.6 for the PCNA index, the benign and LMS were predicted with an overall accuracy of 92% and 82%, respectively. Hence, we conclude that the MIB-1 and PCNA indices are useful for the diagnosis of LMS.

The p53 gene encodes a 53-kDa nuclear phosphoprotein involved in the negative regulation of cell growth (30,31). The mutations in this gene play an important role in the development of many malignant tumors (32-36). Mutant p53 proteins have a longer half-life than the wild-type proteins and are easily detected by immunohistochemical methods. Several investigators have demonstrated an overexpression of the p53 protein immunohistochromically in a variety of human malignancies (15,16). However, no large series of SMTs with p53 evaluation have yet been published. We found p53 protein was expressed in eight of 24 LMS (33%) but in none of the benign SMTs (p = 0.0016). In addition, the p53 positivity statistically correlated with the prognosis of the LMS (p = 0.0357). Recently, mutant p53 has been stressed to be related to cell growth (37) and deviation from apoptosis (38,39). Because the LMS showed cell proliferation more rapidly than that of SMTs as demonstrated with our MIB-1 and PCNA indices study, p53 might only be expressed in the LMS. It is expected that the degree of cell proliferation will correlate with biological behavior of LMS (4-6,40,41). Although our studies did not demonstrate a correlation between the mitotic count, MIB-1 or PCNA index, prognosis, and p53 positivity, in our opinion the p53 expression detects cases with a poor prognosis. p53 might thus be expressed in tumors with a high cell proliferation rate that could not be detected by mitotic count or MIB-1 or PCNA index in the LMS. Therefore, the
p53 expression is a good marker for predicting potential cases with LMS. A semiquantitative evaluation for muscle-specific actins in uterine SMTs was compared with their described urogenital myomas as being strongly positive and two LMS as being negative. Thus, if there is little or no staining with an actin, an SMT should be regarded with caution. Malignant cells appear to be heterogeneous and lose differentiation for contributing their cases for this study. We also thank Kouhei Akazawa (Department of Medical Informatics, Faculty of Medicine, Kyushu University) for assistance with statistical analysis and Brian T. Quinn for editing the manuscript. This study was supported by a Grant-in-Aid for Scientific Research (No. 06280105) from the Ministry of Education, Science, and Culture, Japan.

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