A new scoring system using multiple immunohistochemical markers for diagnosis of uterine smooth muscle tumors

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Abstract

The diagnosis of uterine smooth muscle neoplasms by light microscopy is difficult. Multiple classification schemes have been proposed based on mitotic rate, nuclear atypia, and the presence or absence of necrosis. None of these classification systems has been entirely successful. This study was undertaken to evaluate the use of selected immunohistochemical and histochemical markers in differentiating these tumors, in addition to accepted morphologic criteria. Ten cases of each of the following: leiomyosarcomas (LMS), atypical leiomyomas (AL), cellular leiomyomas (CL) and usual leiomyomas (UL), were classically evaluated for histological diagnosis and were stained for Ki-67 (MIB-1), bcl-2 and p53 using monoclonal antibodies and the avidin-biotin peroxidase method, and argyrophilic nucleolar organizer region (AgNORs). The number of stained cells was counted in the most positively stained region in a 4 mm² square cover glass mounted on each slide. The mean value was calculated for each group of tumors. The data for Ki-67 (MIB-1), bcl-2, p53 and AgNOR staining respectively, were significantly higher in LMS by comparison to UL, CL or AL. Because many singular cases had superimposed data being difficult to diagnose, a new scoring system for pathological evaluation was created. The results obtained by this scoring system suggest that immunohistochemical markers Ki-67 (MIB-1), bcl-2, p53 together with the AgNOR staining could be useful, by the scoring system, as an adjunct to the current accepted morphologic criteria in differentiating smooth muscle tumors of the uterus.

Keywords: uterus • leiomyosarcomas • atypical leiomyomas • cellular leiomyomas • usual leiomyomas • immunohistochemistry • score

Introduction

Leiomyomas are common benign smooth muscle tumors occurring in nearly 40% of women older than 35 years of age. The types in terms of histological criteria consist of usual leiomyoma (UL), cellular leiomyoma (CL), atypical leiomyoma (AL), and smooth muscle tumors of uncertain malignant potential (STUMP). In contrast, leiomyosarcomas (LMS) are rare tumors accounting for 0.21-6% of all smooth muscle tumors of the uterus with a peak incidence in the fifth decade [1]. The risk of local recurrence and metastasis is high in LMS and the reported 5 year...
survival rate in cases of uterine LMS is only 12–25% [2].

The diagnosis of benign, low malignant potential and malignant uterine smooth muscle tumors depends on the extent of occurrence of mitotic figures, nuclear atypia and necrosis. In 1994, Peters et al. [3] studied a group of 47 women with LMS and STUMP which met the criteria of Hendrickson and Kempson [4] for STUMP. The STUMP group had a better prognosis than LMS group but in their study too there were some of these tumors that had a very aggressive course [3]. The distinction between leiomyomas, smooth muscle tumors of low malignant potential and LMS may at times be problematic. While the majority of smooth muscle neoplasms arising within the myometrium are either obviously malignant with atypical nuclear features and high mitotic index, or are clearly benign with bland nuclear features and little or no mitotic activity, there remains a significant group of smooth muscle neoplasms with intermediate degrees of mitotic activity and nuclear atypia [5]. In our department there were not enough cases of STUMP so we could not include them in this study.

Another group which provides a diagnostic problem is clinically benign smooth muscle tumors with gross, cytological or architectural features suggesting malignancy. This group includes leiomyomas with increased mitotic index, CL – hypercellular tumors that resemble LMS since LMS also possess an increased number of cells per unit area. This group also contains AL – benign tumors with focal or diffuse collections of pleomorphic cells including giant cells [4].

Prediction of behavior in this subset of smooth muscle neoplasms has proved to be difficult using simple morphologic evaluation. Various schemes have been proposed to predict the behavior of these neoplasms. In most proposals, the mitotic figures’ index is the primary determinant for distinguishing leiomyoma from LMS [6, 7]. The mitotic index is usually expressed as the number of mitotic figures per 10 high power fields [6]. Although counting mitoses would appear to be an entirely objective procedure, many factors can affect the final mitotic count [4–9]. Some authors have used a combination of mitotic activity and nuclear atypia to improve the accuracy of the diagnosis. Kempson and Bari [10] developed a system based on mitotic activity and the presence or absence of significant nuclear atypia.

Because simple morphologic evaluation of haematoxylin-eosin stained sections has been equivocal in the prediction of behavior in some uterine smooth muscle neoplasms, various ancillary techniques have been evaluated to improve diagnostic accuracy. Among these are proliferation markers AgNOR, Ki-67, p53 protein expression, and expression of the oncogene bcl-2 [1–5, 11–13].

In general, the proliferation markers AgNOR, and Ki-67 (MIB-1) have shown significant differences between mean values for LMS and benign smooth muscle tumors [1, 12–16]. The results of p53 analysis have been variable with the predominance of data supporting a diagnostic utility for this marker [1, 5, 14, 17]. There is limited information about bcl-2. Bcl-2 protein was expressed in nearly all benign smooth muscle tumors but in only 57% of the LMS [2, 18].

In this study we wanted to check the usefulness of these markers to distinguish between benign and malignant uterine smooth muscle neoplasms, by creating a scoring system helpful in deciding for individual cases.

Materials and Methods

Patients

Specimens from 40 patients with smooth muscle tumors of the uterus which were retrieved from the files of the Department of Pathology, Rabin Medical Center, Golda Campus, Petah-Tikva, Israel were examined.

Among these were 10 cases each of LMS, AL, CL and UL. In each case the histopathologic diagnosis was given according to the classification scheme of Hendrickson and Kempson [5]. In addition, AgNORs, expression of Ki-67 (MIB-1), p-53 and bcl-2 were assessed in each case.

AgNOR staining technique

AgNOR staining was performed as reported by Ploton et al. [19]. 4 μm thick sections of routinely processed formalin-fixed, paraffin embedded blocks were cut and
placed on SuperFrost®Plus slides (Menzel-Glaser, Braunschweig, Germany). The slides were oven dried overnight at 60°C, then deparaffinised in xylene and following this, hydrated through 3 decreasing concentrations of ethanol to distilled water. The AgNOR staining solution was composed of 2% solution of gelatin in 1% formic acid. One volume of this solution was then mixed with two volumes of 50% aqueous silver nitrate solution to obtain the final solution. The working solution was then directly applied to the sections and left for 60 minutes in the dark, at room temperature. The silver colloid was then washed off with deionized water, and the slides were dehydrated through graded concentrations of alcohol to xylene and then mounted in pertex mounting media.

Counting procedure for AgNOR

The AgNORs were visualized as intranuclear black dots. In all specimens the most positive stained region was selected. We covered this area with a glass on which a square of 4 mm² was marked [20]. We counted the dots inside this square at 400x magnification. The total number of dots for each slide was counted. In each group the mean value of counted black dots was calculated.

Immunohistochemical staining technique

Sections, 4 µm thick, of routinely processed formalin–fixed, paraffin embedded blocks were cut, and placed on SuperFrost®Plus slides. The slides were oven dried overnight at 60°C, deparaffinised in xylene and then hydrated through 3 decreasing concentrations of ethanol to distilled water. Afterwards the slides were treated in citrate buffer solution, pH = 6, Zymed Corporation (San Francisco, CA), ready for use, for 10 minutes in a microwave oven at 92°C. Following this process the slides remained in the citric buffer solution for a 20 minute cooling period. The slides were then washed in a Ventana (Arizona, USA) wash buffer solution and put inside the Ventana. The Ventana was activated by loading the preprogrammed recipe file for the appropriate antibody. Immunohistochemical staining was performed by the avidin - biotinylated peroxidase complex technique using the following antibodies, coupled with the DAB detection kit of Ventana. The antibodies used in this study was obtained from the Zymed Corporation (San Francisco, CA) and diluted for staining as follows: anti-Ki-67 (MIB-1) – 1:20; anti-bcl-2 – 1:300 and anti-p53 – 1:100.

Counting procedure for immunohistochemistry

A similar counting procedure as described above for AgNOR staining was used for immunohistochemistry result analysis. The stains for Ki-67 and p53 are nuclear stains. The bcl-2 stain is a cytoplasmic one resulting in a fine brown cytoplasmic granularity. The stained cells appear to have brown nuclei. In all specimens the most positively stained region was selected. The total number of stained cells for each immunohistochemical marker tested for every slide was counted. The mean value of stained cells was calculated for each group.

Proposed new scoring system for diagnosis of uterine smooth muscle tumors

The scoring system means to calculate the sum of the values obtained for all four stains (AgNOR, Ki-67, bcl-2 and p53 in a standard area of 4 mm² of the highest stained area) in every case. The average values for these new values were used to determine a quite confident border in order to appreciate the leiomyoma type, by histological criteria, in every singular case.

Statistical methods

Statistical significance was analyzed using student’s t test, and found to be highly significant for every pairs of data sets specific for every individual staining used in our study.

Results

The clinico-pathological data are summarized in Table 1. The results of all stains as number of stained cells/4 mm², mean ± SD and scores are shown in Table 2. Statistical analysis showed a high significance by comparing every pairs of data group.
As shown in Table 2, the count of NORs, in the 4 mm² square for AgNOR technique in the LMS group, resulted in an average value of 257.3 ± 157.5 (the highest count was 569 and the lowest 46). For AL group, the highest value was 97 and the lowest 13, with a mean value of 37.6 ± 27.6. The highest count in the CL group was 67 and the lowest 12; average value = 35.3 ± 22. Similar results were obtained in the UL group, with 72 the highest count and 15 the lowest (mean value = 36.4 ± 17.2). The highest count in the benign cases was 97, (case number 10 in the AL group), 67 in the CL group and 72 in the UL group. In the LMS group there were two exceptional low cases: number 3, with a value for AgNOR count of 46 and number 1, with 69. In conclusion, by AgNOR staining, even though in many cases the differences are easily visible and helpful for pathologists, there are also cases with critical values difficult for confident diagnosis.

**Ki-67** is a marker for cell proliferation used to evaluate a proliferation index for tumor cells. In our study Ki-67 immunohistochemical staining was used for counting positive cells. For LMS group Ki-67 staining (Fig. 1A) gave values of 230 as the highest and 15 as the lowest respectively, with an average value of 115.2 ± 79.4. In the AL group Ki-67 immunostaining (Fig. 1B) revealed six negative cases (cases 1 to 6 in Table 2). In one case the count reached 113, an extremely high value, taking into account the average value of 17.3 ± 35.4. In the CL group there were five negative cases (2, 3, 5, 8 and 10 in Table 2). In one case (number 7) the count was 63. The mean value was 12.1 ± 22.9. In the UL three cases were negative (1, 2 and 9 in Table 2). The highest count was 17 with an average of 4 ± 5.4. The highest count of Ki-67 in the benign group was 113 in case number 10 of AL group. The LMS group had four cases with lower counts, value 15 (number 10),

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**Table 1**: Clinical data of patients with uterine leiomyosarcomas, atypical leiomyomas, cellular leiomyomas and usual leiomyomas

| Clinical data | LMS Mean ± SD | AL Mean ± SD | CL Mean ± SD | UL Mean ± SD |
|---------------|---------------|--------------|--------------|--------------|
| Age Mean     | 61.2 ± 11.2   | 39.33 ± 3.85 | 44.44 ± 5.49 | 44.4 ± 9.94  |
| Range        | 50-84         | 29-53        | 37-54        | 24-63        |
| Number of nodules |   |             |              |              |
| One          | 5             | 6            | 2            | 1            |
| Two          | 1             | 1            | 3            | 2            |
| >2           | 4             | 3            | 5            | 7            |
| Maximal diameter (cm) | | | | |
| Mean         | 8.81 ± 4.05   | 9.05 ± 4.71  | 5.85 ± 2.57  | 6.1 ± 2.33   |
| Range        | 4–15          | 1.5–18       | 2–9          | 2–9          |

Leiomyosarcomas = LMS; Atypical Leiomyomas = AL; Cellular Leiomyomas = CL; Usual Leiomyomas = UL
Table 2  Number of stained cells/4mm², mean ± SD and scores

| Leiomyosarcomas | AgNOR | Ki 67 | bcl2 | p 53 | Score |
|-----------------|-------|-------|------|------|-------|
| 1               | 69    | 141   | 73   | 117  | 400   |
| 2               | 281   | 20    | 32   | 73   | 406   |
| 3               | 46    | 185   | 23   | 193  | 447   |
| 4               | 315   | 30    | 86   | 60   | 491   |
| 5               | 167   | 128   | 109  | 111  | 515   |
| 6               | 234   | 51    | 196  | 62   | 543   |
| 7               | 417   | 178   | 8    | 115  | 718   |
| 8               | 178   | 174   | 152  | 247  | 724   |
| 9               | 297   | 230   | 212  | 165  | 904   |
| 10              | 569   | 15    | 186  | 172  | 942   |
| Average ± SD    | 257.3±157.5 | 115.2±79.4 | 107.7±75.5 | 131.5±61.6 | 609±200.4 |

Atypical leiomyomas

| 1               | 19    | 0     | 4    | 7    | 30    |
| 2               | 22    | 0     | 0    | 15   | 37    |
| 3               | 19    | 0     | 3    | 16   | 38    |
| 4               | 13    | 0     | 23   | 12   | 48    |
| 5               | 17    | 0     | 16   | 26   | 59    |
| 6               | 21    | 0     | 6    | 37   | 64    |
| 7               | 62    | 14    | 0    | 4    | 80    |
| 8               | 49    | 12    | 4    | 22   | 87    |
| 9               | 57    | 34    | 26   | 0    | 117   |
| 10              | 97    | 113   | 0    | 4    | 214   |
| Average ± SD    | 37.6±27.6 | 17.3±35.4 | 8.2±9.8 | 14.3±11.5 | 77.4±54.9 |

Cellular leiomyomas

| 1               | 12    | 2     | 10   | 0    | 24    |
| 2               | 27    | 0     | 2    | 2    | 31    |
| 3               | 12    | 0     | 14   | 8    | 34    |
| 4               | 36    | 9     | 0    | 3    | 48    |
| 5               | 46    | 0     | 0    | 4    | 50    |
| 6               | 66    | 1     | 8    | 16   | 91    |
| 7               | 17    | 63    | 0    | 19   | 99    |
| 8               | 67    | 0     | 32   | 6    | 105   |
| 9               | 55    | 46    | 0    | 7    | 108   |
| 10              | 15    | 104   | 57   | 127  | 176   |
| Average ± SD    | 35.3±22.0 | 12.1±22.9 | 17±32.2 | 12.2±16.9 | 76.6±47.8 |

Usual leiomyomas

| 1               | 19    | 0     | 0    | 0    | 19    |
| 2               | 25    | 0     | 8    | 7    | 40    |
| 3               | 29    | 5     | 6    | 2    | 42    |
| 4               | 41    | 1     | 5    | 0    | 47    |
| 5               | 53    | 2     | 0    | 3    | 58    |
| 6               | 47    | 4     | 3    | 16   | 70    |
| 7               | 72    | 2     | 1    | 0    | 75    |
| 8               | 30    | 9     | 3    | 46   | 88    |
| 9               | 33    | 0     | 0    | 63   | 96    |
| 10              | 15    | 17    | 58   | 27   | 117   |
| Average ± SD    | 36.4±17.2 | 4±5.4 | 8.4±17.6 | 16.4±22.2 | 65.2±29.7 |
value 20 (number 2), value 30 (number 4) and value 51 (number 6). Again cases difficult to diagnose appear.

**Immunostaining for bcl2**

Bcl-2 is an antiapoptotic factor highly expressed in tumor cells and is an alternative marker for immunohistochemical diagnosis. For bcl2 immunostain in the LMS group (Fig. 2A) the highest count was 212, the lowest was 8, and the average value was $107.7 \pm 75.5$. In the AL group three cases were negative (2, 7 and 10 in Table 2) and the highest count reached 26; average value $= 8.2 \pm 9.8$. In the CL group four cases were negative (4, 5, 7 and 9 in Table 2), while one case reached the considerable high value of 104, for an average of $17 \pm 32.2$. All the cases of the UL group had very low counts (three were negative), except for one case that showed 58; average value $= 8.4 \pm 17.6$. There was one case, in the CL group, with an exceedingly high count, 104 (number 10). In the LMS group there were five cases with lower counts by comparison with CL case number 10 mentioned above; these are: value 8 for case 7, value 23 for case 3, value 32 for case 2, value 73 for case 1 and value 86 for case 4. Therefore, using bcl-2 immunostaining several cases are also difficult to diagnose.
Staining for p53

Antigen p53 is another marker for tumor diagnosis, but it is also expressed at high level in many actively proliferating, nontransformed cells. In our study, the highest count for p53 staining in the LMS group was 247, while the lowest was 60, with an average value of 131.5 ± 61.6. Staining for p53 in AL group (Fig. 2B) revealed one case completely negative (number 9 in Table 2). The highest count reaching a value of 37 was noted for case number 6, and the average value was 14.3 ± 11.5. Similar counts were achieved in the CL group with one negative case (case number 1 in Table 2) and one with 57 positively stained cells. The average value for CL group was 12.2 ± 16.9. Three cases were negative in the UL group (1, 4 and 7 in Table 2). The highest count was 63 and the average value was 16.4 ± 22.2. In the LMS group there were two cases with lower counts, 60 (number 4) and 62 (number 6). As for the above described markers, for p53 immunostaining critical values for diagnosis appear too.

New scoring system usage

To avoid the inconveniences due to the existence of many cases with superimposing results for every of the individual staining procedures, and trying to find a way to reach an accurate diagnosis, we created a new set of data as a result of summation of values obtained for all staining procedures per case. These new figures were used as score values and an average value was calculated for every type of uterine smooth muscle tumors. The highest score values were: 942 for LMS group, 214 for AL group, 176 for CL group and 117 for UL group respectively; the lowest score values were: 400 for LMS group, 30 for AL group, 24 for CL group and 19 for UL group respectively. Therefore, the score values in all 10 cases of LMS group were much higher than the score values in every other benign group. The statistical analysis between the LMS group and each of the benign groups resulted in a statistical significance with one order of magnitude higher than the results obtained analyzing data for individual staining (not shown). But the real advantage of the scoring system is pointed out under Discussion.

Discussion

Morphologic evaluation based on hematoxylin and eosin-stained sections is an imperfect predictor of behavior for uterine smooth muscle neoplasms [5]. In those few cases in which the biological behavior is uncertain, additional methods of immunohistochemistry and molecular biology have to be applied. In this study we used a set of immunohistochemical and histochemical stains which usually showed clear differences between the LMS group and that of the benign groups. But sometime in single cases there were some exceptional results with all stains. Moreover, statistics is not at all helpful for pathologists in such cases. The data, resulting in counting slides processed by various (immuno)histochemical techniques used in this paper, gave by analysis high significance if considering the cases from the
statistical point of view. This is not the case for pathologists and clinicians working with individual cases. This paper introduces a new scoring system to differentiate between these cases. The highest score value in the benign groups was 214 while the lowest in the LMS group was 400. Therefore, we suggest a cut-off point at 250 (horizontal red line in Fig. 3) that is about 15% higher than the highest score value in the benign group and almost 35% lower than the lowest score value in LMS group.

Many authors have investigated alternative techniques for the prediction of biological behavior in uterine smooth muscle tumors. Proliferation markers AgNOR, Ki-67, p53 protein expression, and expression of the oncogene bcl-2 all have been studied in an attempt to improve prognostication for these neoplasms [12–17].

Several immunohistochemically demonstrable antigens, including the proliferating cell nuclear antigen (PCNA) and Ki-67 (MIB-1), are known to correlate with the number of cells actively proliferating [17, 21]. A significant correlation was found between the mitotic rate, the mitotic index and Ki-67 in cases of LMS [14]. Our results are in agreement with other previous studies which report increased Ki-67 expression in uterine LMS in contrast to leiomyomas [1, 14, 15].

Mutations in the p53 gene and p53 protein accumulation have been found in a wide range of human malignancies of both epithelial and mesenchymal origin [22]. Our results are in agreement with majority of the studies which have demonstrated little or no staining for p53 in leiomyomas. Mutations and over expression of p53 have been reported in LMS of the uterus [22–24]. The majority of reports indicate that the presence of increased amounts of immunohistochemically detectable p53 protein product supports a diagnosis of LMS, but the absence of such staining does not exclude aggressive behavior [15, 23, 24].

Bcl-2 expression has been demonstrated in a variety of normal tissues as well as human cancers [18, 21]. In their study, Zhai et al. [2] revealed the presence of a higher bcl-2 expression level in benign uterine smooth muscle cell tumors (UL, CL, bizarre leiomyoma – BL and STUMP), than in normal myometrium, or in LMS. In LMS 43% were partially positive, 14% were diffusely positive for bcl-2 while 43% were negative analyzing 21 cases [2]. Soini et al. [18] found extensive bcl-2 immuno-

positivity in malignant smooth and striated muscle tumors, while in benign tumors the immuno-positivity was more inconsistent. Our results are more consistent with the latter study only in that notable significant differences in the number of cells stained for bcl-2 were observed between benign and malignant tumors in our data.

We found, as expected, that there was a statistically significant difference in the mean number of stained cells between the malignant lesions and the benign smooth muscle tumors for all of these stains. Because for many individual cases there were superimposing data difficult for diagnosis we created a new scoring system helpful for pathologists. To our knowledge this is the first study to use a scoring system based on proliferating markers in the differential diagnosis of uterine smooth muscle tumors.

Although the number of cases studied here was limited, we think that the new scoring system may be useful as an additional tool to the common histological criteria used for discriminating ALs, CLs, and ULs from LMSs. We think that the new scoring system will allow every laboratory in pathology to design its own scattering diagram and cut-off point according to specific needs.

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