

## Original Article

### Diagnostic Value of Proliferatin Index Including MIB1 and Argyrophilic Nucleolar Organizer Regions Proteins in Uterine Smooth Muscle Tumors

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#### ABSTRACT

**Background and Objectives** Uterine smooth muscle tumors are the most common human neoplasm. They are divided clinically as benign and malignant but there is another group of lesions which is difficult to place in these two categories, so-called smooth muscle tumors of uncertain malignant potential (STUMP) and differentiation of these tumors on the basis of H&E staining is impossible. The goal of this study was finding of distinguishing objective biomarkers and a survey of proliferation markers comparing these three groups of tumors.

**Materials & Methods:** Twenty one cases in each group of above mentioned tumors were selected randomly (63 cases in total ) from Pathobiology laboratories and studied by using immunohistochemistry (IHC) staining for Ki-67 expression and special Argyrophilic Nucleolar Organizing Regions (AgNOR) staining method.

**Results :** Ki-67 was expressed in 63.15% of leiomyosarcomas (LMS), 4.76% of STUMPs and 0% of leiomyoma group. Ki-67 expression between LMS and STUMP, and also between LMS and leiomyoma were significantly different ( $P < 0.0001$ ). Mean AgNOR dots were  $2.55 \pm 0.03$ ,  $2.55$ ,  $0.66$ ,  $4.04$ , and  $8.12 \pm 0.13$  in leiomyoma, STUMP and leiomyosarcoma, respectively. Significant differences between the three groups were observed ( $P < 0.0001$ ).

**Conclusion:** AgNOR and Ki-67(MIB1) proliferation markers expression between LMS and STUMP were significantly different. Due to difficulty in differentiation LMS from STUMP, finding objective biomarkers is useful and practical. For this purpose, the present study recommend, Ki-67 and AgNOR staining which is a reliable, simple and rapid method.

**Keywords:** Nucleolus Organizer Regions, Smooth Muscle Tumors, Uterus, Ki-67 Antigen

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## Introduction

Uterine smooth muscle tumors are one of the most common tumors in human. The majority of these tumors are readily classifiable into benign or malignant. There are some lesions, however, for which that placement is very difficult, sometimes impossible so-called "smooth muscle tumors of uncertain malignant potential (STUMP)". The benign uterine smooth muscle tumors, leiomyomas, are perhaps the most common neoplasm of female genital tract and may be present in about 75% of females of reproductive ages (1,2). The malignant counterpart of those lesions, leiomyosarcoma, on the other hand, is relatively infrequent with aggressive behavior.

STUMPs are difficult to categorize and composed of few tumors, but they occasionally can recur and metastasize to distant sites. According to the literature, most of these tumors, have a benign clinical course, but requiring long-term, close follow-up. This fact emphasizes the precise differentiation of smooth muscle tumors of uterus (1-3).

At present, the diagnosis of uterine smooth muscle tumors is by light microscopic examination. 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 (4-8).

Nucleolar organizer regions (NORs) are loops of DNA included ribosomal genes, from which ribosomal RNA is translated in order to synthesis proteins. NORs are in short arm of acrocentric chromosomes and associated with argyrophilic proteins, which are seen as black dots, termed "AgNOR" by using colloidal silver (Ag) staining method. AgNOR count in nucleus have direct relation with cellular proliferative activity (9-11).

Because simple morphologic evaluation based on hematoxylin and eosin-stained sections is an imperfect predictor of behavior in some uterine smooth muscle tumors, various ancillary techniques have been evaluated to improve diagnostic accuracy. Among these, proliferation markers, including silver-staining nucleolar organizer regions (AgNORs), and Ki-67 (MIB-1), have been investigated (4, 8, 12).

The diagnostic value of these studies has varied from report to report. In general, Proliferation markers have shown significant differences between mean values for prognostically favorable and unfavorable groups of uterine smooth muscle tumors (8,12,13).

The present study was designed to evaluate the potential value of AgNOR staining and Ki-67 immunohistochemical marker in differentiating different types of uterine smooth muscle tumors.

## Materials and methods

### Selection of patients:

The formalin-fixed paraffin-embedded tissues of sixty one cases of uterine smooth muscle tumors from the files of Pathology Department, Imam Reza and Zeinabieh Hospitals of Kermanshah and Shiraz Universities of Medical Sciences (Iran), from 2001 to 2008, were selected.

Selected cases were divided into three groups according to the work of Richard Kempson criteria and diagnostic terms (1) for uterine smooth muscle tumors as following; leiomyoma (n=21), smooth muscle tumors of uncertain malignant potential (STUMP, n=21) and leiomyosarcoma(n=21).

The diagnosis was agreed upon by two experienced independent pathologists, based on the examination of conventional hematoxylin-eosin staining. Two cases of LMS excluded from the study because the diagnosis were not confirmed by two pathologists. A representative block was selected for each case. The slides were processed both for immunohistochemistry and AgNOR staining.

### AgNOR Staining method :

Formalin-Fixed paraffin-embedded sections (3-5 ~~mm~~ thickness) were rinsed in xylene for 5-10 minutes three times, in ethanol (100%) for 2-5 minutes three times, then in ethanol (70%) for 2-5 minutes once. The slides were washed in running tap water for 5 minutes and were then rinsed in deionized water for 2-3 minutes 2-3 times. The slides were incubated with 1 volume of solution A and 2 volumes of solution B per slide. The slides were left in the dark for 60 minutes and rinsed in deionized water three times. In addition, the sections were dehydrated through a graded series of ethanol to xylene, and the prepared smears were mounted with a cover slip.

### Solution A (Colloid Developer Solution )

Solution A contained 100ml of pure water, plus 2 g of gelatin, plus 1ml formic acid. The gelatin was dissolved at room temperature or at 40°C for several months.

### Solution B

Solution B contained 100ml of pure water, plus 50g of silver nitrate. This solution has to be stored in the dark by wrapping aluminum foil around the container.

### AgNOR Quantitation:

Silver-stained slides were examined with the aid of a 1000x magnification with an oil-immersion lens. AgNORs appear as brown or black dots within a yellowish background of nucleus.

The quantitation were performed in well preserved cells, excluding areas of tumor necrosis, staining artifacts or overlapped cells. First, the brown /black dots were counted in the nuclei of 100 cells/case and the mean number of dots was taken for each case (mAgNOR). A mean of 10 different areas of tumor was chosen in order to determine the homogeneous AgNOR quantitation throughout the tumor. The second count was the percentage of nuclei exhibiting five or more AgNOR granules/nucleus/100 cells called proliferative index (pAgNOR). This count was believed to represent proliferative activity.

Sections stained to show AgNOR, were examined blindly by two pathologists, independently. The AgNOR Proliferative index (pAgNOR) were calculated by counting the cells having five or more AgNOR granules per nucleus, in 100 Nuclei. The results were analysed statistically by one- way ANOVA and t-test.

### Immunohistochemical staining for Ki-67 (MIB-1):

Additional 3-5 tissue sections of the same blocks, used for AgNORs method were cut, mounted on slides, deparaffinized in xylene, rehydrated and retrieved by steamer in tris-buffered saline (pH=9) for 40 minutes. The slides were immunostained using mouse monoclonal antibody for Ki-67 (Dako, Carpinteria, Calif), and the avidin-biotin-peroxidase method. Antibody were from clone MIB-1 (N1633). The final reaction product was developed with diaminobenzidine. The remainder of the staining procedure was performed and also positive and negative tissue controls were used as previously described utilizing the Dako universal mouse kit. All cases with nuclear staining of tumor cells were considered positive. The data were analyzed statistically using the Chi-square tests to compare the frequency distributions of Ki-67 expression between

the groups (leiomyoma, STUMP and LMS).

P values of less than 0.05 were considered statistically significant. The SPSS system (SPSS 15.0, Inc.) was used for the calculations.

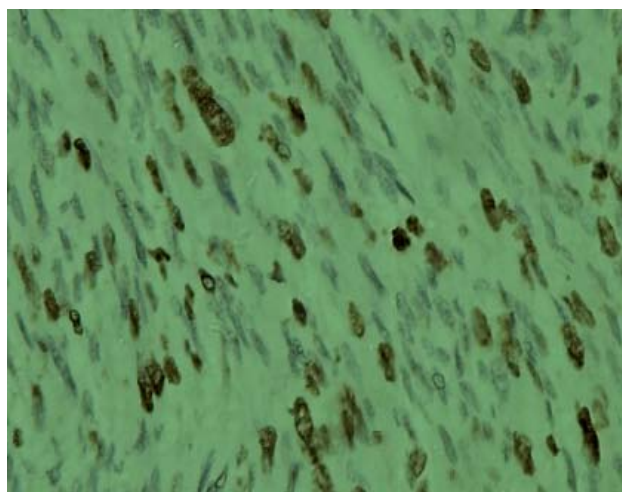
## Results

Sixty-one cases of uterine smooth muscle tumors, including 21 leiomyoma, 21 STUMP and 19 LMS cases, were examined in this study. Black silver-stained dots for AgNOR were clearly identified in cell nuclei of three groups of tumor.

The mean AgNOR count per cell in 21 cases of leiomyoma was  $2.55 \pm 0.03$ . In 21 Cases of STUMP, the mean AgNOR count per cell was  $4.04 \pm 0.66$ . The mean AgNOR count in 19 cases of leiomyosarcoma was  $8.12 \pm 0.13$ . The pAgNOR count in uterine smooth muscle tumors increased from leiomyoma to leiomyosarcoma (8% in leiomyoma, 39% in STUMP and 86% in LMS). This count was believed to represent proliferative activity.

The AgNOR dots were discrete and smaller in benign cases as compared to coarse and aggregated in malignant cases.

The AgNOR count in these 3 groups showed a statistically significant difference ( $P < 0.0001$ ). Multiple comparisons also showed also demonstrated a statistically significant difference in the AgNOR count in each of the three groups, when we looked separately (leiomyoma and STUMP ( $P < 0.0001$ ), leiomyoma and LMS ( $P < 0.0001$ ), and also STUMP and LMS ( $P < 0.0001$ ), therefore, there was no overlap between the 3 groups (Fig. 1).



**Fig. 1:** Immunostaining of Ki-67 in leiomyosarcoma. Note predominant nuclear immunostaining pattern (magnification  $\times 400$ )

In immunohistochemical assessment, Ki-67 proliferation marker was present in 12/19 (63.15%) leiomyosarcoma cases, in 2/21 (4.76%) STUMP cases and in 0/21 (0%) leiomyoma cases.

Significant differences regarding the frequency of

ki-67 expression were observed between LMS and STUMP ( $P<0.0001$ ) as well as between LMS and leiomyomas ( $P<0.0001$ ), but not between STUMP and leiomyomas ( $P=0.48$ ) (Table 1).

**Table 1:** Comparison of mean, standard deviation (SD) and proliferative index of AgNOR in three groups of uterine smooth muscle tumors.

Uterine smooth muscle tumor	mAgNOR count per cell (mean $\pm$ SD)	pAgNOR Proliferative Index (percentage of nuclei with $\geq 5$ AgNOR dots/nucleus)
Benign	2.55 $\pm$ 0.03	8%
Uncertain malignant potential	4.04 $\pm$ 0.66	39%
Malignant	8.12 $\pm$ 0.13	86%

## Discussion

Morphologic evaluation based on hematoxylin and eosin-stained sections is an imperfect predictor of the behavior for uterine smooth muscle neoplasms. In those few cases in which the biological behavior is uncertain, additional methods such as immunohistochemistry or special histochemical staining method may be helpful(5).

In this study we used an immunohistochemical and a histochemical stain which mainly showed obvious differences between the LMS group and that of the benign group.

Nucleolar organizing regions (NORs), are loops of rDNA placed in the nucleolus which play an important role in the synthesis of ribosomes and gene proteins. It has been determined that the number and the size of NORs are related to the proliferative activity and grade of malignancy. Furthermore, in recent years, AgNOR has been used to in order to distinguish between malignant and benign neoplasms (14-20). This study demonstrated that the mean number of AgNOR dots increases for malignant uterine smooth muscle tumors as compared to benign types.

AgNOR staining results showed significant difference between three groups of uterine smooth muscle tumors ( $P<0.0001$ ), as previous studies have also reported (5). Therefore, it has been determined that the amount of nucleolar organizer regions argyrophilic proteins markedly increases in the neoplastic process revolution toward malignancy (13, 20-22).

In our study, the mAgNOR and pAgNOR

(proliferative index) were high in more proliferative and mitotically active tumor cells. We therefore proposed that mAgNOR would probably be the reflection of the total number of chromosomes or ploidy, and the percentage of cells with five or more AgNOR granules in 100 cells (pAgNOR) would reflect proliferative activity, as already reported by Mourad et al.(23-25).

More recently, some studies have demonstrated a positive correlation between AgNOR and other markers of cellular proliferation, such as Ki-67 index (10;12;26). Ki-67(MIB-1) immunohistochemically demonstrable antigen, is known to correlate with the number of cells actively proliferating (5). In a standardized morphometric analysis of AgNORs in breast carcinoma it was found that AgNOR expression was significantly higher in cycling (MIB1 positive) tumor cells, than in resting (MIB1 negative) ones, however with certain exceptions (12, 23, 24).

Our results are in agreement with other previous studies which report increased Ki-67 expression in uterine LMS in contrast to leiomyomas (27-32). However it should be noted that the Ki-67 expression between STUMP and LMS groups was significantly different( $P<05$ ).

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. A positive correlation was found between Ki-67 and AgNOR in cases of LMS (8, 28, 33, 34).



## Conclusion

As the differential diagnosis between STUMP and leiomyosarcoma may be in some cases problematic, using an objective biomarker that would allow unambiguous identification and thereby improve diagnostic specificity would be enormously helpful. For this purpose we suggest Ki-67 immunohistochemical marker and AgNOR staining, which is reliable, simple, rapid and cheap method, in differentiating uterine smooth muscle tumors with benign behavior from those of malignant.

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