Evaluation of KI-67 expression in uterine leiomyoma and in healthy myometrium: a pilot study

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INTRODUCTION

Uterine fibroids or leiomyomas are benign tumors that originated from the smooth muscle of the uterus. Their estimated incidence reaches 75% of childbearing-age women. They are characterized histologically by areas of disordered growth of smooth muscle fibers and extracellular matrix. These tumors arise naturally during the reproductive age. They are usually related to biological changes in growth and...
development mainly influenced by sex hormones, especially estrogen, in addition to other causes such as genetic changes and growth factors.

Leiomyomas can have a significant impact on the quality of life of women in reproductive age. Depending on their anatomical position, quantity, and size, these tumors can cause menstrual irregularity in about 30% of cases, with hemorrhage being the most frequent symptom. Increased uterine volume may induce an elevated pelvic pressure, leading to pain and compression of other structures, such as the rectum and bladder, causing constipation and urinary incontinence. Moreover, uterine fibroids have a negative impact on reproductive function and are associated with infertility and adverse gestational outcomes, such as miscarriages, fetal anomalies, premature births, and an increase of indications for cesarean sections.

Due to its high prevalence in the population and its impact, its pathogenesis must be fully understood in order to develop better therapeutic strategies. The aim of this study was to evaluate the expression of the KI-67 cell proliferation marker in uterine leiomyomas and adjacent myometrial tissue. Furthermore, this paper aims to verify the existence of a connection between clinical parameters and KI-67 expression in tumors.

METHODS
Type of study and sample design

This study is part of a research project whose goal is to evaluate several markers (KI-67, BCL-2, IGF-1, among others) and their clinical correlations in the symptomatology of uterine leiomyomatosis. A significant sample size of 60 was determined based on the number of patients treated at our service. Because this is a pilot study, it was decided to reduce the sample to 10 to analyze the viability of the research. In addition, we selected KI-67 as the first marker to be studied due to the greater experience of the pathology team with this marker. In addition, its qualitative analysis is simpler when compared to the others.

This is a cross-sectional, controlled, and analytical study. This study included ten women with symptomatic uterine leiomyomatosis who underwent a total abdominal hysterectomy at a tertiary hospital in Teresina-PI. They were randomly selected by batch from March 2017 to December 2017. The inclusion criteria were: women over 18 years of age with symptomatic uterine leiomyomatosis and a surgical indication of a total hysterectomy by laparotomy. The exclusion criteria were: menopausal women, patients with a cancer diagnosis or with clinical suspicion of malignancy, previous hormone therapy or surgical intervention for uterine fibroids.

Collection of samples

After the hysterectomy, two tissue samples were collected of one-centimeter diameter. One sample was composed of uterine leiomyoma and the other one of myometrial tissue. The healthy myometrium was located at least five centimeters away from any leiomyoma. Then, the samples were submitted to the following procedures: 1) Formalin fixation; 2) Dehydration through ethyl alcohol; 3) Diaphonization in xylol; 3) Impregnation with paraffin at an oven temperature of 59 º C. After this process, part of the samples underwent the consecutive serial cuts of four micrometers. Next, they were kept on glass slides and stained with hematoxylin and eosin for diagnostic confirmation of normal myometrial tissue (figure 1) and uterine leiomyomatosis (figure 2).

In addition, the following data were collected from medical records: age, gestational age, fibroids with greater volume, uterine volume, intensity of menstrual bleeding (mild, moderate, or severe), post-operative hemoglobin, Body Mass Index (BMI) and ethnicity. Due to the intense menstrual irregularity that some participants presented, it was not possible to accurately assess their stage of the menstrual cycle.

Immunohistochemical method

A monoclonal antibody to KI-67 was used. The samples were dewaxed in xylol at 110 º C and, soon after, subjected to multiple washouts with water at...
room temperature. After this, the cuts were placed in ethyl alcohol at concentrations 100, 80, and 50 percent consecutively. Then, they were washed in running tap water. Lastly, the sections underwent distillation. Endogenous peroxidase activity was blocked with hydrogen peroxide (H2O2) 3% three times for 10 minutes each. This last step was followed by washing the samples with Phosphate-Buffered Saline (PBS) solution (pH 7.4 to 7.6). In order to unmask antigens, the slides were boiled at 95°C for 30 minutes in sodium citrate buffer solution (pH 6.0) in a T-fal streamer. After cooling for 20 minutes, the sections were washed in tap and distilled water and, ultimately, placed in PBS. The incubation time with a specific primary antibody was overnight at 4°C.

After incubation, the slides were washed three times in PBS, dried and incubated with «EnVisionTM System (DAKO, Code K 1672)» for 1 hour at 37 °C. Once again, the sections were washed in tap and distilled water and then stained with Mayer’s hematoxylin for 30 seconds. The cuts underwent alcohol-xylol dehydration and, lastly, placed on coverslips with Entellan resin.

**Samples evaluation**

The expression of the biomarkers was evaluated by two blinded observers with no information regarding sample identification. These observers counted the number of cells with positively stained nuclei under 400x magnification using an optical microscope attached to a video camera.

For the quantification of Ki-67 biomarker expression, 500 cells were counted on each slide. The percentage of stained cells was calculated from the ratio of the number of cells with stained nuclei and the total number of cells multiplied per 100, as in the formula below:

\[
\text{Percentage of stained cells} = \left( \frac{\text{cells with colored nuclei}}{\text{Total number of cells}} \right) \times 100
\]

**Statistical analysis**

The data were tabulated using Microsoft Excel 16.0 and summarized in tables and graphs; Student’s t-test was used for statistic comparisons. The significance level was set at p<0.05. Pearson’s correlation coefficient was used for paralleling the clinical findings and the Ki-67 expression according to the following parameters:

- 0.9 to 1.0 indicates a very high correlation.
- 0.7 to 0.9 indicates a high correlation.
- 0.5 to 0.7 indicates a moderate correlation.
- 0.3 to 0.5 indicates a low correlation.
- 0 to 0.3 indicates a negligible correlation.

**Ethical aspects**

The research was approved by the Research Ethics Committee of our institution under protocol number 2.061.409. All patients signed an informed consent form.

**RESULTS**

A total of 9 patients were included in the study because one of the samples was not considered satisfactory. The mean age was 40.7 years, ranging from 35 to 44 years. Regarding ethnicity, eight patients were African American, and one was white. The mean size of fibroids was 5.4 cm, ranging from 2.1 to 10.5 cm. The mean uterine volume was 345 cm³ ranging from 127 to 449 cm³.

Three (33.3%) patients classified the pain as mild, and six (66.7%) as severe. Bleeding was considered mild in three (33.3%) patients and intense in the remainder (66.7%). Only three patients had preoperative

**FIGURE 2.** PANEL A: UTERINE LEIOMYOMA (400X MAGNIFICATION). PANEL B: UTERINE LEIOMYOMA WITH CELLS EXPRESSING KI-67 (40X MAGNIFICATION). PANEL C: UTERINE LEIOMYOMA WITH CELLS EXPRESSING KI-67 (400X MAGNIFICATION)
hemoglobin lower than nine; in one case, it was necessary to perform a perioperative blood transfusion. Two patients were nulliparous, two had two gestations, and five became pregnant three or more times. The mean BMI was 24.2, ranging from 21.3 to 29.1; therefore, five patients had adequate BMI (19-24.9), and the remainder were overweight.

Regarding KI-67 expression, there were positively stained cells in all samples included (Figure 2). The mean expression of KI-67 in the myometrium was 1.63%, and, in leiomyomas, it was 5.96% (p <0.001). The correlation between clinical parameters and KI-67 expression in leiomyomas is presented in Table 1.

**TABLE 1. CORRELATION BETWEEN KI-67 EXPRESSION IN UTERINE LEIOMYOMAS AND CLINICAL PARAMETERS ACCORDING TO PEARSON’S CORRELATION COEFFICIENT.**

| Variable                | P    |
|-------------------------|------|
| Age                     | -0.46|
| Parity                  | -0.04|
| Tumor size              | 0.12 |
| Uterine volume          | 0.21 |
| Pain                    | 0.55 |
| Bleeding                | 0.55 |
| Preoperative hemoglobin | -0.55|
| Body Mass Index (BMI)   | 0.05 |
| Weight                  | 0.1  |

**DISCUSSION**

Uterine leiomyomas are benign neoplasms of the smooth muscle of the uterus. They are usually asymptomatic, although they may manifest as abnormal uterine bleeding, pelvic pain, and various combinations of other symptoms that may impair the quality of life of women. They account for 30% of hysterectomies in women during reproductive age and 9.4 billion dollars in medical expenses in the United States.

In recent years, there has been a significant advance in the knowledge of the biology of leiomyomas, but the fundamental mechanisms of their formation are not yet fully understood. More than 200 altered genes have been reported in these tumors, although a few remain prevalent in several populations. Several signaling pathways may also be altered, reflecting the complexity of this neoplasm. It is believed that the probable origin of this neoplasm derives from injuries and successive repairs of the myometrium. Therefore, we can state that, initially, non-hormonal factors are responsible for the onset of the myoma. In a second moment, estrogen and progesterone would play their role in allowing tumor growth.

Classically, leiomyomas are described more frequently in African American women. In addition, the symptomatology is more intense in this group. Other contributors, such as nulliparity, use of progesterone-based contraceptives, obesity, hypertension, and early menarche, have also been associated as risk factors for this disease. On the other hand, the use of combined contraceptives and a diet rich in vegetables seem to exert a protective effect. In fact, some of these agents do not have their mechanisms of action completely clarified, but they can act by deregulating specific pathways, allowing a greater cellular proliferation.

KI-67 is a biomarker that has been researched for years in oncology. It is used to assess the degree of proliferation of a given tissue. When the nucleus of the cell binds to the antibody and produces a characteristic coloration, it indicates that the cell is advancing in the cell cycle. This marker is useful because it allows evaluating the aggressiveness of a tumor: the higher the number of positive cells, the higher the cell proliferation index. Thus, tumors with a greater expression of KI-67 are classically described as more aggressive.

At this moment, a few studies have focused on the expression of KI-67 in fibroids. They usually focus on using it only as a reference to distinguish fibroids from malignant neoplasms. As a result, it is not known how the greater or lesser expression of this marker may influence the presentation or prognosis of uterine leiomyomatosis. The authors concluded that the rate of cell proliferation is lower in myomas than in uterine sarcomas, which is a useful parameter in cases of unclear diagnosis. In our study, KI-67 expression was significantly higher in fibroids than in healthy myometrium. In addition, we found a moderate correlation between KI-67 expression and the reported severity of anemia, level of pain, and bleeding. The value of 0.55 for the correlation between KI-67 expression, pain, and bleeding suggests that the greater the expression of the marker, the greater the intensity of these clinical parameters. Even though the values of correlation of this study are not considered high
(0,7 – 1), they are calculated in a small sample size, suggesting that there is an important relationship among the data studied in this article. Comparatively, the value of - 0.55 calculated for the correlation between KI-67 and preoperative hemoglobin indicates that the higher the marker expression, the lower the level of hemoglobin. The presence of a negative correlation between these two parameters is probably due to the greater bleeding intensity in patients with higher KI-67 expression.

Due to the low number of cases included in this initial study, it is still very early to state that the cell proliferation index is a prognostic factor to be incorporated into clinical practice. However, the results suggest that it probably relates to higher symptomatology. Furthermore, we can conclude that the methodology of this article can be applied to analyze a large number of patients and that the sample size should be increased in order to improve the statistical analysis.

CONCLUSION
The expression of KI-67 in normal myometrium was significantly lower than in leiomyomas. The highest expression of KI-67 was moderately related to the severity of anemia, bleeding, and pain level in the patients of this study.

Author’s Contributions
Concept – Walberto Monteiro Neiva Eulálio Filho, Benedito Borges Silva, Pedro Vitor Lopes Costa; Design – Maria Simone Oliveira Lima, Rodolfo Myronn de Melo Rodrigues; Supervision – Eduardo Augusto Soares Sousa, Benedito Borges Silva; Resources – All authors; Materials – Emerson Davi do Nascimento Brazil, Pedro Vitor Lopes Costa; Data Collection and/or Processing – Walberto Monteiro Neiva Eulálio Filho, Benedito Borges Silva, Eduardo Augusto Soares Sousa; Analysis and/or Interpretation – All authors; Literature Search – All authors; Writing Manuscript – All authors; Critical Review – All authors; Final Review – All authors.

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