

Endometriosis of the Abdominal Wall - Clinical, Histopathological and Immunohistochemical Aspects

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Endometriosis is a benign gynecological pathology that mostly affects the organs of the pelvis, but also organs located at a distance maybe affected. Tissue immunohistochemically identified as ectopic endometrium may undergo certain structural and functional changes that may lead to preneoplastic alterations (hyperplasia, dysplasia) in normal cells which can evolve to neoplasia. Clinical, genetic, metabolic conditions and local factors may influence degeneration of a benign pathology into a malignant pathology. Endometriosis of the abdominal wall is more frequently encountered, as the number of caesarean section has increased. Endometriomas surrounding tissue has a direct impact on the structure of the cells that form the mass. By remodeling cellular morphology, corroborated with the hormonal factors action and the inflammatory response (via lymphocyte cell secretion), the cell cycle is altered and antiapoptotic activity may be promoted. Immune system via lymphocyte cell secretion, the pressure exerted on the tumor area by surrounding tissues with its size change, conditioned by the fluctuation of hormonal factors, act directly on the cellular structure and can increase anti-apoptotic action and decrease cell cycle regulation. The presence of endometriomas is identified by the positivity of immunohistochemical reactions for estrogen receptors (ER), progesterone receptors (PR), Cytokeratin 7 (CK7) for endometrial tissue. Negative reaction at Cytokeratin 20 (CK20) shows that the studied area is not a metastasis of a digestive tumor. The presence of abundant inflammatory, peritumoral cells marked with anti-CD68 / Tryptase for macrophages / mast cells demonstrates the involvement of the inflammatory system in the structural and functional modification of endometrial cells. The pronounced cell division was demonstrated by intense reaction with the anti-Ki67 antibody. The significant anti-apoptotic action of the endometrial tissue is shown by the positivity of anti-B cell Lymphoma 2 (BCL2) / anti-Phosphatase and tensin homolog (PTEN) / anti-p53 antibodies.

Keywords: endometriosis, progesterone receptor, estrogen receptor, Bcl-2, PTEN

Ectopic endometrial implants can be present in the pelvic organs: ovaries, uterine ligaments, rectovaginal septum, peritoneum and extra pelvic structures like kidneys, lymph nodes, pleura, lungs, brain and abdominal wall [1, 2].

Patients experience period-related pain. Tumor's size was significantly bigger before menstruation. This increase amplifies the symptoms. In the time between menstruations, these patients were asymptomatic and they did not report any vaginal bleeding.

In areas of focus, the presence of a large number of ER and PR was demonstrated, in addition to an increasing hormone secretion, causing an abnormal activity of steroidogenesis [3, 4].

The aims of this study were: to identify the presence of ectopic endometrial tissue, to evaluate the inflammatory reaction around it and to observe the preneoplastic (hyperplastic) transformation of the areas of interest.

Experimental part

This retrospective study included a total of 20 patients, who were admitted in the Obstetrics-Gynecology II Clinic of the Craiova County Emergency Clinical Hospital, between 2010-2018. The endometriosis diagnosis was based on the patients' history (all of them had undergone c-section),

their intense symptoms and the presence of endometriomas in their ultrasound examination. The final diagnosis was established after the excision and the biopsy of the endometriomas was performed and the histopathological results were available. All the patients included in the study signed the acceptance form (approved by the Ethics Commission of the University of Medicine and Pharmacy of Craiova) for inclusion in the study after having been provided in writing and verbally with all necessary information. The subjects were divided into two groups: one group formed of 10 patients diagnosed with post-caesarean section abdominal wall endometriosis without preneoplastic alterations and another one composed of 10 patients affected by atypical endometriosis - hyperplastic glands.

Patients' age ranged from 18 to 57 years old and the premalignant condition was diagnosed on older subjects, compared to the benign endometriosis (Chart 1).

Following excision surgery, the tissue was fixed in 10% formalin solution and embedded in paraffin wax as it follows: after fixation, the tissue was washed in tap water for one hour to remove the fixation solution. Then the tissue was immersed into successive alcohol baths 70, 90, 96%, for one hour each and it was left overnight in absolute

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alcohol bath in order to remove tissue's water. The next day, the samples were passed successively through 3 xylene baths, one hour each, and then introduced into the molten paraffin, at 56 ° C, placed in the thermostat, until the next day, so the paraffin penetrates the tissue. On the third day, the solid paraffin block was made and it was cut using the HMB350 microtome equipped with a water-based transfer system (STS microM). The 5 µm sections were applied on clear glass slides and poly-L-lysine coated slides for the histological and immunohistochemical study. For the Haematoxylin and Eosin staining, the sections were deparaffinized in xylene for 45 minutes, hydrated in successive alcohol baths with decreasing concentration: 100, 96, 90, 70%, three distilled water baths, of 5 min each, then the nuclei were stained with the Mayer's Haematoxylin solution (crystallized haematoxylin 1.0 g, distilled water 1000 mL, sodium iodide (NaIO₃) 0.2g, aluminum sulphate 50.0g, citric acid 1g, chlorohydrate 50g). The samples kept for 1 min in this solution and the nuclei were colored in red-violet. To neutralize the solution and stain the nuclei blue, the colored slides were introduced into tap water for a few minutes (the alkaline content from water neutralizes acidity of aluminium haematoxylin to form the blue aluminium haematin complex). Subsequently, the cell cytoplasm and collagen fibers were dyed with Floxin-Eosin solution (*Stock solution Eosin Y*: eosin:1g, distilled water -100mL, alcohol 96% -300mL. Eosin was dissolved in warm water and, after cooling, alcohol was added; *Stock solution Floxin*: floxin: 1g, distilled water -100 mL, alcohol 96% -300mL. To dissolve the floxin, the solution should be warmed up; *The Eosin-Floxin Work Solution*: Eosin stock 450mL + Floxin stock 50 mL + Glacial Acetic Acid 2 mL) for a few minutes.

For the immunohistochemical study, the sections were dewaxed and rehydrated similarly, a 'magic circle' was drawn around the tissue sections using a Dako Pen, a water repelling solution and then the slides were boiled in the

microwave oven (650W), while submerged in Citrate solution pH=6 [5, 6] (Sodium Citrate 29.4g, HCl 1M 29.4g, distilled water 1L, and adjusting the pH to 6 with 1M HCl. Further on, for the stock solution of Citrate buffer (10x) pH 6 to expose the antigen, we used Citric Acid Monohydrate 21.014g and distilled water 1000mL, following the pH adjustment with NaOH and 1:10 dilution with distilled water at the time of use) / EDTA pH=9 (Tris-EDTA: we used ethylenediaminetetraacetic acid 0.372g, distilled water 1000mL and adjusting the pH to 9 with NaOH and HCl), 7 cycles x 3 min for antigen unmasking. After cooling, the slides were washed in 3 distilled water baths for 5 min each. Afterwards the blocking of nonspecific sites (endogenous peroxidase) was achieved using diluted Perhidrol solution (2 mL of 30% perhidrol, 100mL distilled water) for 30 min, and washing the slides in 2 distilled water baths for 5 min each in Phosphate-buffered saline (PBS) solution (NaCl 0.12 M- 13.92g, NaH₂PO₄ · 2H₂O 0.01M - 3.12g, K₂HPO₄ 0.04M -13.92g, distilled H₂O 200mL) [7]. Subsequently, the slides were sunk in 3% skimmed milk powder solution 3g powdered milk + 100mL PBS) for 30 min. The last step of the first day is the addition of the primary antibody to the sections (table 1) and leaving the slides in a wet container for 18 h at a temperature of -4 ° C. The following day the cold slides were brought at room temperature, washed in PBS for 5 min 3 times, then the secondary antibody was applied (Mouse/Rabbit IgG Antibody, VC002-025, R&D Systems, VisUCyte HRP Polymer), which remained for 1 h, then they were developed with 3,3'-Diaminobenzidine (DAB) (Dako). The reaction is blocked in PBS, slides are washed in distilled water, and the nuclei are counterstained using Mayer's Haematoxylin. After that, the sections are dehydrated with increasing alcohols (70, 90, 96, 100%) and then, they are clarified in three xylene baths (15 min each) and covered with cover glass using Canada balm.

Anti-body	Manu-facturer	Clone	Antigenic exposure	Secondary antibody	Dilution	Labeling
Anti-CK7	Dako	OV-TL 12/30	Citrate, pH 6	Monoclonal mouse anti-human cytokeratin 7	1:50	Glandular epithelia
Anti-CK20	Dako	Ks20.8	Citrate, pH 6	Monoclonal mouse anti-human cytokeratin 20	1:25	Cellular protein of mature enterocytes and goblet cells
Anti-ER	Dako	1D5	EDTA, pH 9	Monoclonal mouse anti-human estrogen receptor alfa	1:50	Estrogen receptors α
Anti-PR	Dako	PgR 636	EDTA, pH 9	Monoclonal mouse anti-human progesterone receptor PR	1:50	Progesterone receptors
Anti-p53	Dako	DO-7	EDTA, pH 9	Monoclonal mouse anti-human p53 protein	1:50	Nuclear marker
Anti-BCL2	Dako	124	EDTA, pH 9	Monoclonal mouse anti-human BCL-oncoprotein	1:50	B-cell lymphoma 2
Anti-PTEN	Abcam	ab31392	Citrate, pH 6	Polyclonal Rabbit	1:50	Tumour suppressor gene
Anti-CD68	Dako	KP1	Citrate, pH 6	Monoclonal mouse anti-human CD68	1:100	Macrophages
Anti-Tryptase	Dako	AA1	Citrate, pH 6	Monoclonal mouse anti-human mast cell tryptase	1:500	Mast Cells

Table 1
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At the end, the sections were imaged utilising a Nikon Eclipse 55i microscope equipped with a 5MP CCD color cooled camera and the Image ProPlus 7 analysis software (Media Cybernetics). All images were stored as uncompressed tiff files, and the DAB signal on each immunohistochemistry slide was quantified as the number of units (cells). The values were then averaged per patient and then the data were compared utilising a Student's t test. P values <0.05 were deemed significant.

Results and discussions

The age of the women included in the study are between 18 and 57 years old and it was noticed that precancerous cellular changes (endometrial hyperplasia) occur with a higher frequency in elderly patients (Chart.1)

All patients complained about chronic pelvic pain.

Through histochemistry we established the diagnosis of endometriosis. Using classic histological Haematoxylin Eosin staining of the tissue sample we observe the simple columnar tissue, the basal located nuclei, and the cytogenetic chorion placed around the endometrial glands (fig 1.)

Immunohistochemical staining with anti-ER and anti-PR antibodies facilitated the identification of the abundance of hormone receptors, which demonstrates the strong involvement of estrogen and progesterone in the evolution of endometriomas (fig 2,3).

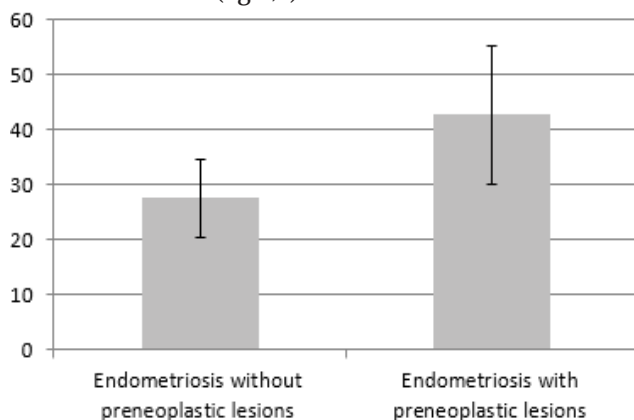


Chart.1 Age of diagnosis of endometriosis

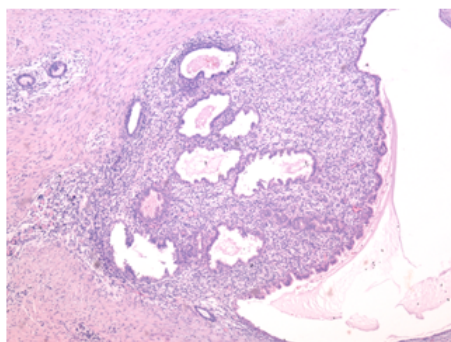


Fig. 1. Haematoxylin Eosin staining, 100 \times . The endometrial glands are observed among the smooth muscle fibers in the abdominal wall.

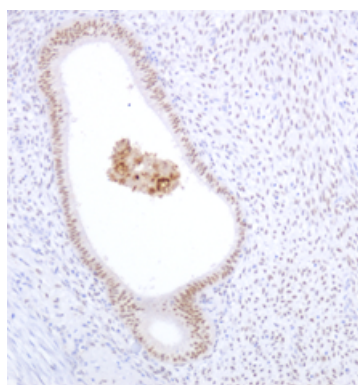


Fig. 2. Anti-ER antibody immunostaining, 200 \times . Positive reaction to ER is observed in the epithelia and lamina propria.

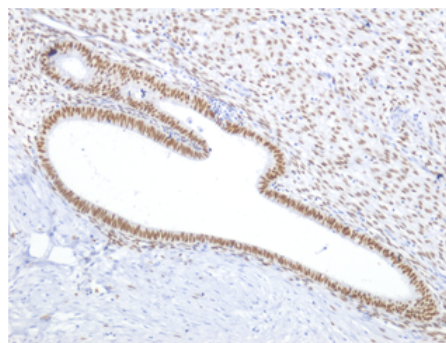


Fig. 3. Anti-PR antibody immunostaining, 200 \times . Positive reaction to PR is observed in the epithelia and lamina propria

The first malignant transformation of endometriosis was described in 1925 by Sampson [7], and the prevalence of this transformation is 0.7-1.6% [8]. The most common malignant tumor types are: clear cell carcinoma, endometrial carcinoma, various types of endometrial stromal sarcomas, Muleterian tumors, or borderline tumors and benign tumors [9].

Recent studies have suggested that some genetic mutations at the ER, p53 or BCL2 level in this phenomenon may be involved [10-12].

ER is part of a group of intracellular proteins. These receptors are activated by the estrogenic hormone (17 β -estradiol) [13]. After activation, the ERs are translocated into nuclei and sets the DNA to regulate the activity of several genes (transcription factor of the DNA) [14]. ER α that is found in the endometrium, mammary carcinoma cells, ovarian stromal cells and in the hypothalamus [15]. ER α se are found in the endometrium, mammary carcinoma cells, ovarian stromal cells and in the hypothalamus [15]. In males, ER α is present in the epithelium of the eferent ducts [16].

PRs are part of the nuclear receptor subfamily 3rd and are activated by the progesterone hormone. PRs are encoded by the PGR gene on chromosome 11q22 [17, 18].

By immunolabeling with anti-CK 7 and anti-CK20 markers, we were able to differentiate endometrial glands from other structures such as an adenocarcinoma's metastasis originating in the digestive tract.

All factors involved in the etiopathogenesis of endometriosis induce cellular alterations that may promote a malignant transformation of the ectopic endometrial tissue. Thus normal ectopic cells become hyperplastic cells, that may also evolve into dysplastic cells and subsequently transform into neoplastic cells (Chart 2)

CK7 is a protein encoded by the KRT7 gene [19-20], of type II. It is particularly expressed in the epithelium of cavitory internal organs, glandular ducts and blood vessels. The gene coding type II CK is located on chromosome 12q12-q13. These may have other locations, but not all have been fully described. CK7 is expressed by glandular and transient epithelia. It is also found in neoplastic cells, which is why immunohistochemistry differentiates transitional cell carcinomas from ovarian cells. It is used in conjunction with Cytokeratin 20 (CK20) for the differential diagnostic [21] (fig 4, 5).

Bcl-2 is part of the Bcl-2 family of regulatory proteins, which modulate cell apoptosis by either inducing or inhibiting it [22, 23]. Bcl-2 is located on the outer membrane of the mitochondria and plays an important role in regulating the dynamics of the mitochondrial enzymes. Genetic changes of Bcl-2, by unbalancing cell death and

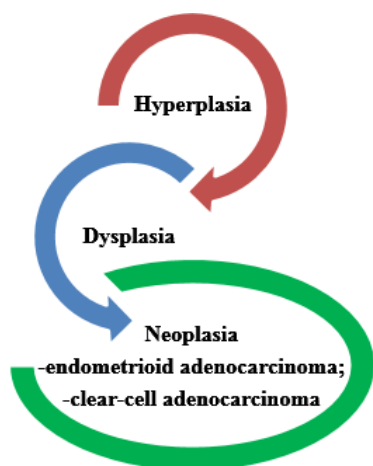


Chart 2. Endometrial cells changes.

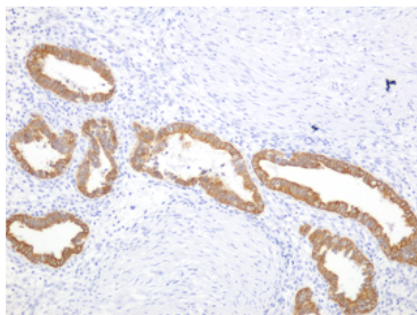


Fig. 4. Anti-CK7 antibody immunostaining, 200 \times . Intense signal can be observed in the epithelia.

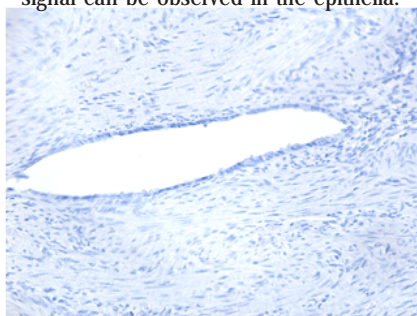


Fig. 5. Anti-CK2 antibody immunostaining, 200 \times . Negative reaction of the endometrial glands is illustrated

cell growth, are associated with various malignant diseases such as prostate cancer, breast cancer, chronic lymphocytic leukemia, lung cancer. Positive immunostaining with the anti-Bcl-2 antibody reveals marked ectopic endometrial cells, which may influence their transformation into hyperplastic, dysplastic and subsequently neoplastic cells. Using the anti-B-cell lymphoma 2 (BCL2) antibody, we demonstrated the involvement of an anti-apoptotic protein that plays an important role in regulating cell death (fig. 6).

PTEN is a human protein encoded by the PTEN gene [24], and its mutations are linked to the development of multiple cancers.

PTEN acts as a tumor suppressor gene via a phosphatase protein product. Normally, it blocks the cells' cycle so that they will not grow and will not divide too fast [25]. PTEN gene's mutations may influence the cell cycle, which leads to an accelerated division. During tumor's growth, PTEN mutations or deletions are produced by enzymatic inactivation, thus increasing cell proliferation and decreasing the rate of apoptosis. It is frequently inactivated in endometrial cancer, prostate cancer and it is reduced in many other cancers. The anti-Phosphatase and tensin homolog antibody (PTEN) marks a protein encoded by the PTEN gene (fig. 7). In conclusion, PTEN mutations cause a predisposition to malignancy.

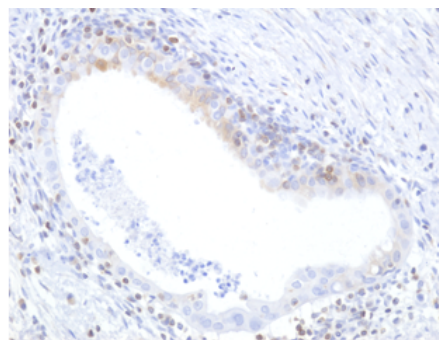


Fig. 6. Anti-Bcl-2 antibody immunostaining, 200 \times . Positive reaction to Bcl-2 is seen.

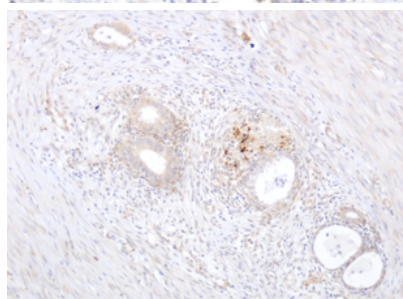


Fig. 7. Anti-PTEN antibody immunostaining, 100 \times . Positive reaction to PTEN is observed

Tryptase is produced by the mast cells and is found in the secretion granules. It is associated with the allergic reaction and it is thought to act like fibroblasts. Some genetic mutations resulting from FIP1L1-PDGFR α fusion may be connected to the neoplastic transformation [26]. By immunohistochemical staining using the anti-Tryptase antibody, we illustrated the presence of many mast cells in the vicinity of the endometriomas, unlike areas situated away from the ectopic endometrial tissue, which were far less populated with mast cells. These cells are closely related with the immune reaction of the endometriomas' surrounding tissue.

CD68 is a protein specific to circulating monocytes and tissue-macrophages (microglia, Kuffer cells) [27]. Immunohistochemical staining detects the presence of CD68 + in cytoplasmic granule macrophages. CD68 is used as a marker for the line of macrophages, monocytes, histiocytes, giant cells, Kuffer cells and osteoclasts. Areas surrounding endometriomas may encounter a significantly higher macrophage count compared to distant areas.

By using anti-Tryptase and anti-CD68 antibodies immunostaining we have demonstrated that there is an intense inflammatory reaction around ectopic endometrial tissue and that

the number of macrophages and mast cells is greatly increased in these areas as opposed to remote areas (fig. 8, 9), (Chart 3, 4).

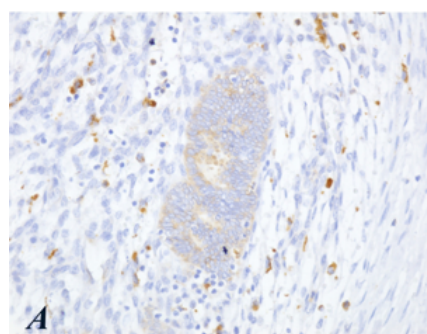


Fig. 8A. Anti-CD68 antibody immunostaining, 200 \times . Positive reaction to CD68 near the endometriosis is observed

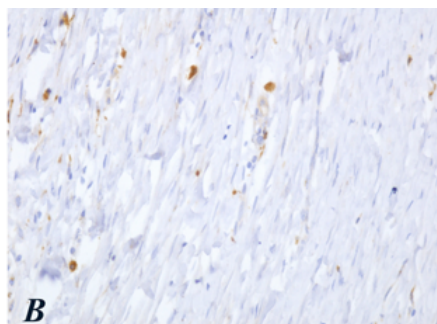


Fig. 8B. Anti-CD68 antibody immunostaining, 200x. Low positive reaction to CD68 away from the endometriosis is observed.

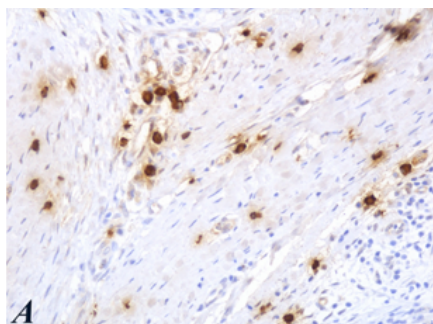


Fig. 9.A. Anti-Tryptase antibody immunostaining, 200x. Positive reaction to Tryptase near the endometriosis is observed; B. Anti-Tryptase antibody immunostaining, 200x. Low positive reaction to Tryptase away from the endometriosis is observed.

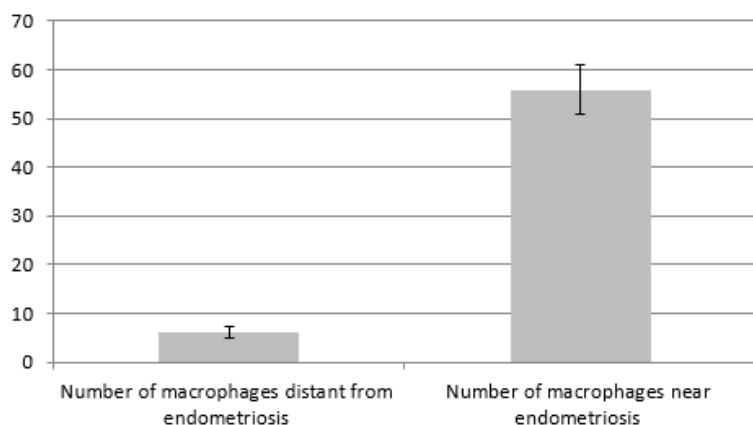
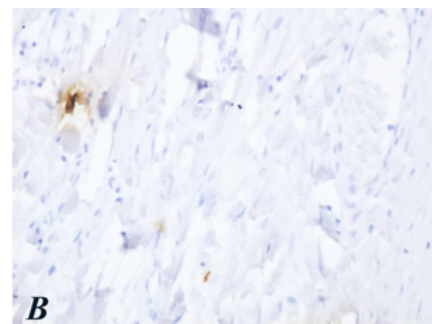


Chart 3. Statistical analysis of the number of macrophages near and away from the endometriosis outbreak. The counting was performed on images obtained with the 200x lens, (statistically significant value: $p < 0.001$).

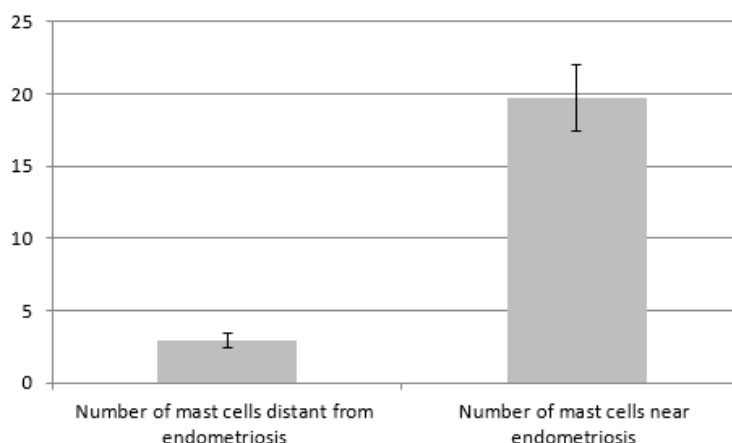


Chart 4. Statistical analysis of the number of mast cells near and away from the endometriosis outbreak. The counting was performed on images obtained with the 200x lens, (statistically significant value: $p < 0.001$).

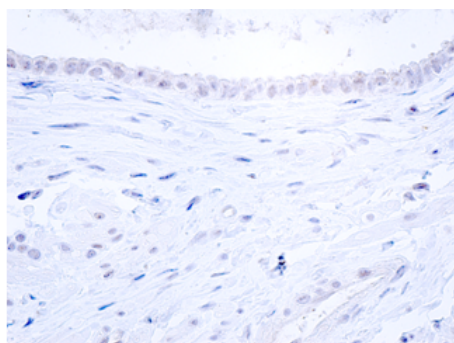


Fig. 10. Anti-p53 antibody, immunostaining 400x. Positive reaction to p53 is observed in endometrial epithelium.

p53 is a cellular tumor antigen that plays a key role in cellular apoptosis, in genomic stability, and in the inhibition of angiogenesis respectively. Therefore it is a multi-action anti-cancer agent: it activates DNA repair [28,29], initiate apoptosis, may block cells in G1 / S phase to provide the time required for the DNA repair. The positivity of p53 immunohistochemical reactions suggests the presence of structural and functional cellular lesions leading to malignant transformation of the cells (fig. 10).

Conclusions

A multitude of inflammatory, hormonal, and mechanical factors are involved in the occurrence and development of endometriosis.

The inflammatory reaction surrounding the ectopic endometrial tissue may contribute to the malignant transformation of it.

Estrogen is involved in the growth and development of glands found in the structure of endometriomas. Decreasing progesterone level creates an unbalance in hormonal status and predisposes to this pathology.

The presence of cellular apoptosis inhibiting genes increases the cell division rate, and the intense immune response can influence the malignant transformation of ectopic endometrial tissue.

The most effective treatment of endometriosis is still surgical removal of the lesions, thus suppressing the pain caused by this pathology and preventing at the same time the malignant transformation of endometriomas.

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