Progesterone and estradiol receptors and Ki-67 in the superficial and deep infiltrating endometriosis

Receptores de progesterona e estradiol e Ki-67 no estroma e no epitélio de endometriose superficial e profunda

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ABSTRACT

Introduction: Endometriosis is a hormone-dependent disease characterized by ectopic presence of endometrial tissue responsive to ovarian steroids. Estrogen and progesterone are the main regulators of endometrial tissue, and the expression of receptors of these hormones in the ectopic tissue seems to be related to the pathophysiology of the disease. Ki-67 is a marker of tissue proliferation and an important marker of epithelial kinetics. Endometriosis can be classified as superficial, in the peritoneum, and deep, when it extends into ligaments and other organs. **Objective:** Our objective was to analyze the expression of estrogen and progesterone receptors and Ki-67, through immunohistochemistry, in different sites of endometriosis tissues (superficial peritoneal/ovarian endometriosis and deep infiltrating endometriosis). **Casuistic and methods:** We studied nine patients; five with superficial and four with deep endometriosis. Statistical correlation was performed with the Shapiro-Wilk test (significance level of 5%) and linear correlation analysis using Spearman's non-parametric test (significance of 1%). There was a correlation of Spearman between the estrogen receptor variable and Ki-67 in patients with superficial endometriosis. There was also a correlation between the variables estrogen receptor and progesterone receptor in patients with deep endometriosis. **Results:** Contrary to what was found for superficial endometriosis, there is linear increase of the variables, with a strong and positive correlation coefficient. This demonstrates that the variation of estrogen receptors can be explained in 99.1% by the same variation of progesterone receptors in deep endometriosis. **Conclusion:** It is possible to infer that other factors are involved in the response to hormonal variations for superficial and deep endometriosis.

Key words: endometriosis; estrogen receptors; progesterone receptors; Ki-67 antigen.

RESUMO

Introdução: Endometriose é doença hormônio-dependente caracterizada pela presença ectópica de tecido endometrial responsivo aos esteroides ovarianos. O estrogênio e a progesterona são os principais reguladores do tecido endometrial, e a expressão de receptores desses hormônios no tecido ectópico parece ter relação com a fisiopatologia da doença. O Ki-67 é um marcador de proliferação tecidual e importante sinalizador da cinética epitelial. A endometriose pode ser classificada em superficial e profunda, atingindo ligamentos e outros órgãos. **Objetivo:** O objetivo deste estudo foi analisar a expressão dos receptores de estrógeno e progesterona e Ki-67, por meio de imuno-histoquímica em endometriose superficial peritoneal/ovariana e endometriose infiltrativa profunda. **Casuística e métodos:** Estudamos nove casos, cinco de endometriose superficial e quatro de endometriose profunda. A correlação estatística foi efetuada com os testes de Shapiro-Wilk (nível de significância 5%) e a análise de correlação linear, pelo teste não paramétrico de Spearman (1% de significância). Houve correlação de Spearman entre a variável receptor de estrogênio (RE) e Ki-67 em pacientes com endometriose superficial e entre as variáveis RE e receptor de progesterona (RP) em pacientes com endometriose profunda. **Resultados:** Ao contrário do que foi encontrado para endometriose superficial, há o aumento linear das variáveis, com coeficiente de correlação forte e positivo. Isso demonstra que a variação dos receptores para estrogênio pode ser explicada em 99,1% pela mesma variação dos RP na endometriose profunda. **Conclusão:** É possível inferir que estejam envolvidos outros fatores nas diferentes respostas hormonais para endometriose superficial e profunda.

Unitermos: endometriose; receptores estrogênicos; receptores de progesterona; antígeno Ki-67.

RESUMEN

Introducción: Endometriosis es una enfermedad dependiente de hormonas que se caracteriza por la presencia ectópica de tejido endometrial sensible a los esteroides del ovario. El estrógeno y la progesterona son los principales reguladores del tejido endometrial, y la expresión de receptores de esas hormonas en el tejido ectópico parece tener conexión con la fisiopatología de la enfermedad. El Ki-67 es un marcador de proliferación tisular y de la cinética epitelial. La endometriosis puede ser clasificada en superficial y profunda, alcanzando ligamentos y otros órganos. Objetivo: El objetivo de este estudio fue analizar la expresión de los receptores de estrógeno y progesterona y Ki-67, mediante inmunohistoquímica en endometriosis superficial peritoneal/ ovárica y endometriosis infiltrativa profunda. Casuística y métodos: Estudiamos nueve casos: cinco de endometriosis superficial y cuatro de endometriosis profunda. La correlación estadística fue realizada con el test de Shapiro-Wilk (nivel de significación del 5%), y el análisis de correlación linear, por la prueba no paramétrica de Spearman (nivel de significación del 1%). Hubo correlación de Spearman entre la variable receptor de estrógeno (RE) y Ki-67 en pacientes con endometriosis superficial, y entre las variables RE y receptor de progesterona (RP) en pacientes con endometriosis profunda. Resultados: Al contrario de lo que se ha encontrado para endometriosis superficial, hay aumento lineal de las variables, con coeficiente de correlación fuerte e positivo. Eso demuestra que la variación de los receptores para estrógeno puede ser explicada en el 99,1% por la misma variación de los RP en la endometriosis profunda. Conclusión: Es posible deducir que otros factores estén involucrados en las diferentes respuestas hormonales para endometriosis superficial y profunda.

Palabras clave: endometriosis; receptores estrogénicos; receptores de progesterona; antígeno Ki-67.

INTRODUCTION

Endometriosis is a chronic estrogen-dependent recurrent condition of multifactorial etiology, characterized by the ectopic presence of endometrial tissue responsive to ovarian steroids[1, 2]. It is histologically identified by the finding of epithelial glandular cells of the endometrium accompanied by endometrial stroma responsive to ovarian steroids to a greater or lesser extent[3].

That functional endometrium of extrauterine regions occurs mainly in structures adjacent to the uterus (pelvic peritoneum and ovaries), but also in structures noncontiguous to tube ostia, as within the substance of the uterosacral ligament, in the rectovaginal septum and, more rarely, in distant sites, as pericardium, pleura, and central nervous system[4].

Nisolle and Donnez (1997)[5] presented the possibility of classifying endometriosis into three forms, from the point of view of etiopathogeny and clinical manifestations: superficial peritoneal endometriosis (SPE), ovarian endometriosis and deep infiltrating endometriosis (DIE). This proposal is based on the hypotheses that explain the development of the disease and its clinical and epidemiological peculiarities. SPE is the most superficial form, arises as lesions on the peritoneal membrane, whose appearance ranges from brown or dark hemorrhagic foci to points in which local peritoneum resorption occurs; the different clinical aspects correspond to different evolution steps of peritoneal endometriosis[5]. Ovarian endometriosis is manifested as cysts of varied sizes called endometriomas; superficial implants can also occur in the ovary, resembling those of the peritoneum[5].

DIE affects approximately 20%-35% of the patients with endometriosis. It is defined as the presence of endometrial implants penetrating structures more than 5 mm in depth, usually involving uterosacral ligaments, rectovaginal septum, muscles adjacent to the uterus and/or invading the pelvic organs[6]. There are many doubts whether these clinical forms of endometriosis are, effectively, distinct aspects of the same disease[7].

Rectovaginal endometriosis is one of the most severe forms of DIE[7]. The endometriosis that reaches the rectum, that which infiltrates the bladder, and adenomyosis are also examples of DIE[8].

Estrogen and progesterone are the main regulators of endometrial tissue. It is estimated that each one of those hormones regulates the expression of thousands of genes, determining the characteristic endometrial changes of the menstrual cycle, by binding and interaction with their respective receptors[2]. The ectopic endometrial tissue of endometriosis foci and the topic endometrium respond to those hormones with evident peculiar histofunctional changes, because both endometrial tissues (topic and ectopic) contain progesterone receptors (PR) and estrogen receptors (ER)[9]. ERs play an important role in survival and maintenance of those tissues, mediating estrogenic action. There are two isoforms: ER alpha and ER beta, apparently with different structures and functions. According to studies, ER beta appears in large quantity in endometriosis tissues, when compared with

topic endometrial tissues, and is associated with high levels of cyclooxygenase-2 (COX-2) and synthesis of prostaglandins in endometriosis, which cause chronic pelvic pain[2].

Progesterone actions are mediated by interaction with its receptor, which can also be expressed in two isoforms: PR-A and PR-B. Those isoforms are transcribed from the same gene, but have different promoters, which confer distinct transcription activities. Although their exact functions are still not fully understood, PR-B seems to be a potent activator of progesterone target genes, while PR-A appears to act as a dominant repressor of PR-B, besides diminishing the response of other steroidal hormones, such as androgens and estrogens[10]. Estradiol acts in endometrium and stromal cells stimulating *PR* gene to transcribe proteins that form progesterone receptors; thus, it makes endometrium responsive to progesterone action. However, PRs in messenger ribonucleic acid (mRNA) and proteins are not high in the biopsy of endometriosis tissues, which are constantly exposed to high levels of estrogen. This indicates that in endometriosis, *PR* gene action in response to estradiol is highly attenuated. Accordingly, high levels of estradiol and the increase of ER beta seem to suppress ER alpha expression[10].

Although estrogen and progesterone hormone receptors are commonly expressed both in topic endometrial tissues and in ectopic endometrial foci[1], knowledge about the influence of those receptors in the progression of endometriosis is still not wide, so further studies can make an important contribution to the treatment of the disease. This distinction of steroid actions and effects in different topographies of endometriosis can contribute to the following understanding: whether they are effectively distinct diseases under the point of view of etiopathology and clinical evolution.

Epithelial kinetics can be evaluated by some histological markers, such as apoptotic index, topoisomerase II alpha (TOP2A) expression, p53, c-erb2, and Ki-67. Bassi *et al.* (2015)[11] compared the occurrence of these markers in the topic endometrial epithelium and in DIE, and observed a correlation between lesion size and cellular turnover. Although the apoptotic index is similar in both groups, TOP2A expression was lower in the endometriosis group than in the controls.

Ki-67 is a good marker of epithelial proliferation and its expression in endometriosis tissue seems to suffer the effects of ovarian steroids, because it is over stimulated in patients undergoing ovarian hyperstimulation for in vitro fertilization[12, 13].

Some studies discussed the presence and the concentrations of ER and PR in fragments of superficial and deep endometriosis. However, we did not find any study comparing concentrations of these receptors in different points of the epithelium.

Therefore, we became interested in analyzing the expression of ER and PR, as well as that of Ki-67, in the different sites of endometriosis, comparing specifically peritoneal/ovarian endometriosis with deep endometriosis of the rectovaginal septum in the stromal and epithelial portions.

CASUISTIC AND METHODS

Casuistic

Twenty-four cases were analyzed: 19 were superficial endometriosis and five were deep endometriosis. We selected slides with larger amounts of material and more intact tissue, so that it was possible to conduct histochemical analysis. Samples were paired by patients' age, according to the different sites of involvement by endometriosis. They were stored in the pathology laboratory (Laboratório Stecca de Patologia e Citologia de Sorocaba), and belonged to patients who underwent surgeries. Inclusion criteria were: SPE or DIE diagnosis and existence of enough material in paraffin blocks for the investigation of estradiol, progesterone and Ki-67 receptors. Exclusion criteria were: non characterization of the site affected by endometriosis, inadequate material for immunohistochemical study, and presence of concomitant malignant neoplastic disease. After material selection and application of inclusion/exclusion criteria, nine cases were selected. The study was just begun after a favorable report by the Research Ethics Committee (CAAE 55511616.9.0000.5373).

Methods

Measuring receptors

The immunohistochemistry technique using specific monoclonal antibodies for ER and PR have the advantage of requiring a small amount of tissue that can be fixed and embedded in paraffin[14]. The paraffin blocks were processed and the slides were referred to antigen retrieval. Each slide was recorded at IntelliPATH (Biocare Medical, USA), an automated instrument for immunohistochemical reaction. The negative control was a slide from the same analyzed tissue, in which the primary antibody was replaced by protein buffered saline-bovine serum albumin (PBS-BSA).

Interpreting immunohistochemical analysis

The presence of ER and PR was blindly determined by one of the authors. We quantitatively assessed the number of stained nuclei. We chose to run the count in three different fields, in the epithelial and stromal portion of the specimens, with final

assessment given by the average of the three counts for each site. The fields of interest were chosen in low magnification. With the objective lens of 40×, the expression of the marker was verified in the stained nuclei, after evaluation of 100 cells in each chosen field.

Statistical method

For inferential analysis, initially, normality of variables was checked by the Shapiro-Wilk test (5% significance level). Later, linear correlation was measured by the non-parametric Spearman's test (1% significance); linear regression analysis was performed for significant correlations. For statistical analysis of data and graph production, the softwares Excel, IBM SPSS statistics version 22, 2013 were used.

RESULTS

The SPE cases (five patients) involved peritoneum and ovaries, while the DIE cases (four patients) were found in uterosacral, rectosigmoid, and round ligament.

We tried to separately characterize the expression of ER, PR and Ki-67 in the glandular and stromal portions of the specimens (**Table**).

According to the analysis of coefficients of variation, all variables are heterogeneous, because their coefficient is higher than 20%. In that analysis, a high variation (over 100%) was observed for the stromal and glandular Ki-67 variables in SPE and DIE in the glandular portion, for ER in the glandular portion, and PR in the glandular and stromal portions in DIE.

All those variables presented minimum values equal to zero. There were no outliers detected in the variables analyzed.

From the analysis of normality of variables with the Shapiro-Wilk test at 5% significance level, it is implied that the variables are not distributed according to the normal variable. So, for correlation analysis, the non-parametric Spearman test at 1% significance level was used. Caution was taken to correlate variables ER, PR, and Ki-67 for patients with SPE and, later, with DIE. There was Spearman correlation ($\alpha = 0.01$) among variables ER and Ki-67 in patients with SPE ($r = -0.975, p = 0.005$). This shows that, in this form of endometriosis, with the increase of ER, there is decrease of Ki-67, and vice versa, with a strong negative correlation. By linear regression of data, the linear equation $y = 5.94\text{-}0.1\times$ was obtained, where Ki-67 is the independent variable y and ERs in SPE, the dependent variable, with $r^2 = 0.638$. Those data show that variation of Ki-67 can be explained in 63.8% by variation of ER in SPE.

There was also correlation between variables ER and PR in patients with DIE, with $r = 0.996$ ($p = 0.004$). As opposed to what was found for SPE, there is linear increase of variables, with a strong and positive correlation coefficient. By linear regression of data, the linear equation $y = 16.65 + 1.01\times$ was obtained, where y is the independent variable ER in DIE and PR, the dependent variable, with $r^2 = 0.991$. This demonstrates that ER variation can be explained in 99.1% by the same variation of PR in DIE.

According to the analysis of normality of variables with the Shapiro-Wilk test at 5% significance level, variables were not distributed normally. In agreement with linear correlation analysis for non-parametric variables, no significant correlation was observed.

TABLE – Percentage descriptive analysis of variables ER, PR, and Ki-67 in SPE and DIE in glandular and stromal portions

SPE ($n = 5$)	ER		PR		Ki-67	
	Glandular	Stromal	Glandular	Stromal	Glandular	Stromal
Mean ± SD	30 ± 29.44	32.5 ± 22.17	45 ± 31.09	42.5 ± 20.62	3 ± 4.76	1 ± 1.16
Median	25	30	55	40	1	1
Minimum	0	10	0	20	0	0
Maximum	70	60	70	70	10	2
Coefficient of variation (%)	98.13	68.22	69.09	48.52	158.67	116
DIE ($n = 4$)	ER		PR		Ki-67	
	Glandular	Stromal	Glandular	Stromal	Glandular	Stromal
Mean ± SD	42.5 ± 49.24	32.5 ± 15	10 ± 14.14	39.25 ± 41.5	3.75 ± 4.79	5.5 ± 3.32
Median	40	30	5	37.5	2.5	5
Minimum	0	20	0	2	0	2
Maximum	90	50	30	80	10	10
Coefficient of variation (%)	115.86	46.15	141.4	105.73	127.73	60.36

ER: estrogen receptor; PR: progesterone receptor; SPE: superficial peritoneal endometriosis; DIE: deep infiltrating endometriosis; SD: standard deviation.

DISCUSSION

Considering that some studies showed different functions related to stroma and glands in the genesis of endometriosis, we searched to separately quantify each of these components of the endometriosis tissue[14].

At a study performed by Kamergorodsky et al. (2007)[14], superficial and deep endometrioses presented significant morphologic differences between them, besides the relationship between endometriosis location and cellular differentiation degree.

It is important to study ER and PR owing to the intense influence of those hormones upon the topic endometrial tissue and the endometriosis tissue. It is known that estrogen stimulates proliferation of uterine epithelial cells and progesterone inhibits this estrogenic action, and in endometriosis there is resistance to progesterone action and prevalence of estrogenic action[15]. However, this resistance to the action of progesterone does not imply necessarily a smaller quantity of PR.

Signorile et al. (2009)[16] studied cases of deep endometriosis in the rectovaginal septum and found very similar percentages for the expression of these receptors: the presence of ER ranged from 0% to 94%, while that of PR, from 0% to 94%. Samartzis et al. (2012)[2] also found variable levels of ER alpha and PR in peritoneal, ovarian endometriosis and adenomyosis. Besides, the clinical treatment for endometriosis is based on the exogenous administration of progestogens that are only able to exert their action due to the presence of PRs in a significant amount[17].

There are few articles about the expression of ER and PR in deep endometriosis, and no study was found comparing receptors present in superficial and deep endometriosis. Current knowledge on the action of ERs and PRs in endometriosis is provided by studies about superficial endometriosis.

In our cases, we observed that in SPE, ERs did not associate with the increase of epithelial kinetics marker Ki-67, findings that were statistically significant, but restrict to that form of endometriosis. Our findings were not the same for patients with deep endometriosis.

Zanatta et al. (2015)[15] and Samartiz et al. (2012)[2] studied the expression of ER and PR in DIE, and found strong expression of ER and PR in the glandular and stromal tissue. Such findings differ from those of the literature, whose studies analyzed just cases of superficial endometriosis and concluded that the expression of ER was weak or absent. That divergence in the findings of superficial and deep endometriosis seems to suggest that the physiopathology of these two types of the disease is different, or perhaps the hormones and their receptors can have distinct actions in different environments[15].

Another marker studied by immunohistochemistry in this work was Ki-67, which is widely used as a marker of cellular proliferation. Literature describes it is found in lower levels in the endometriosis tissue than in the proliferative tissue[18].

Calcagno et al. (2011)[19] verified that Ki-67 was expressed in higher levels in proliferative endometrium than in endometriosis tissues and secretory endometrium. This marker is also higher in non-ovarian endometriosis tissues than in ovarian endometriosis. It was also possible to observe a lower expression of Ki-67 in ovarian endometriosis than in endometriosis tissue of other location.

Hormone receptors, as a rule, are expressed in lower quantity in the ectopic endometrium than in the topic endometrium and expressed in lower level in ovarian endometriosis than in non-ovarian endometriosis, as well as in the proliferative endometrium than in the secretory one. This difference of receptors in endometriosis of ovarian and non-ovarian tissue can be explained by the influence of ovarian steroids upon ovarian endometriosis, which would promote down-regulation of receptors in the endometriosis ovarian tissue by means of paracrine effect of ovarian hormones[19].

In this study, DIE was observed to present a larger quantity of undifferentiated cells; therefore with greater capacity for tissue invasion, while SPE presented well-differentiated cells. Our findings were compatible with data in the literature, because in the present study we evaluated the presence of ER, PR and marker Ki-67; they were effectively detected with statistically significant values in the forms of deep endometriosis. Such findings could explain the reasons why DIE manifests more aggressive behaviors and responds less favorably to hormone therapy[8].

We believe our results are important, for they suggest there are really differences from the point of view of hormone action and cellular kinetics in superficial and deep endometriosis, what confirms previous studies, which present similar morbid conditions, but with different populations and natural evolution. It is necessary, therefore, a larger number of cases to confirm the observed tendencies of our study.

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