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ORIGINAL ARTICLE

PI3K/AKT pathway is altered in the endometriosis patient's endometrium and presents differences according to severity stage

Daniela Madanes[a], Mariela A. Bilotas[a], Juan I. Bastón[b], José J. Singla[c], Gabriela F. Meresman[b], Rosa I. Barañao[a]* and Analía G. Ricci[a]*

[a]Laboratorio de Inmunología de la Reproducción, Instituto de Biología y Medicina Experimental – Consejo Nacional de Investigaciones Científicas y Técnicas (IBYME-CONICET), Ciudad Autónoma de Buenos Aires, Argentina; [b]Laboratorio de Fisiopatología Endometrial, Instituto de Biología y Medicina Experimental – Consejo Nacional de Investigaciones Científicas y Técnicas (IBYME-CONICET), Ciudad Autónoma de Buenos Aires, Argentina; [c]Hospital de Clínicas José de San Martín, Ciudad Autónoma de Buenos Aires, Argentina

ABSTRACT

Based on the inflammatory nature and hormone-dependency of endometriosis, PI3K/AKT signaling appears to influence its progression. Could the endometriosis stages be linked to differential changes in PI3K/AKT pathway regulation? The objective is to evaluate the expression of PI3K, PTEN, AKT and p-AKT in endometrial human biopsies, according to the presence or absence of the disease, and to assess the underlying differences regarding the endometriosis stages. Biopsy specimens of the ectopic and eutopic endometrium were obtained from twenty women with untreated peritoneal endometriosis as well as endometrium biopsies from nine controls. Our study revealed an increased expression of PI3K in eutopic and ectopic endometrium from patients with endometriosis, and a reduced expression of PTEN and increased levels of AKT phosphorylation, compared to control endometrium. Both eutopic and ectopic endometrium from patients with minimal-mild endometriosis expressed a significant reduced PTEN level compared to the respective endometrium from patients with moderate-severe endometriosis. The ratio p-AKT/total AKT showed higher levels of AKT phosphorylation in endometriotic tissue from patients with minimal-mild endometriosis. This study has firmly confirmed the alteration in PI3K/AKT pathway regulation and demonstrated clear differences between the stages of endometriosis, emphasizing the importance of this pathway in the first stage of the disease.

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Introduction

Endometriosis is a common gynaecological disorder characterized by ectopically endometrial tissue implantation reassembled as functional endometriotic lesions, frequently disposed in ovaries, peritoneum and rectovaginal septum [1,2]. Population-based data suggest that affects approximately 5% to 10% of reproductive age women [3] and up to 50% of women with infertility [4]. Moreover, emerging data indicate that true prevalence of endometriosis is not satisfactorily established because an estimated 6 of 10 endometriosis cases are undiagnosed [5]. It rarely is asymptomatic [6], being more frequently associated with infertility, dysmenorrhea, dyspareunia, dysuria, and chronic abdominal pain leading to a decreased quality of life [2]. Current evidence of endometriosis social [7,8] and psychological impact on affected women [9,10] shows the imperative need of new and accurate strategies to treat them.

Unfortunately, current treatments are only aimed at alleviating clinical symptoms of the disease. Despite surgical resection of endometriotic lesions is nowadays the primary indicated treatment, recover from this disease became a real challenge considering a recurrence rate up to 50% [11]. Moreover, current hormonal treatments (like progestins, oral contraceptives and gonadotropin-releasing hormone agonists) are estrogen suppressive [12] and do not enable their use as long-term strategies because of their side effects. This led us to looking for non-hormonal targets.

In the last decades, molecular evidence suggested that endometriosis might be triggered by both dysregulation of hormones and/or signaling cascades and cytokines [13]. The phophatidylinositol-3 kinase (PI3K)/protein kinase B (AKT) signaling pathway as well as its major kinases have been identified a long time ago [14], and it is well known that is one of the main intracellular signal transduction pathways that help to maintain the normal physiological cell functions, such as growth, differentiation, metabolism, and apoptosis [15]. Aberrations in this pathway regulation can lead to different proliferative diseases, such as endometrial cancer [16] among others [17–19]. PI3K converts intracellular PtdIns-4,5-P2 (PIP2) to PtdIns-3,4,5-P3 (PIP3). Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase (PTEN) is the major negative regulator of PI3K pathway and is considered as a tumor suppressor in many carcinomas [20]. In the absence of PTEN, the PIP3 lipid product predominates, leading to activation of PI3K-dependent downstream signaling pathways. AKT is one of the primary kinases downstream of PI3K [16].

It has been demonstrated that AKT signaling pathway is hyperactivated in endometrial cancer because of prevalent PTEN mutations [21]. Although endometriosis is defined as a benign disease, certain biological signs -characterized by metastasis,

CONTACT Analía G. Ricci agricci@ibyme.conicet.gov.ar Instituto de Biología y Medicina Experimental, Vuelta de Obligado 2490, Ciudad Autónoma de Buenos Aires C1428ADN, Argentina
*These authors contributed equally to this work.

proliferation and invasion -suggest that it may share some characteristics with the neoplasic process [22,23]. Moreover, it has been described that PI3K/AKT pathway can be activated by estrogen in human endometrium [24]. Based on the inflammatory nature and hormone-dependency of endometriosis, PI3K/AKT signaling appears to influence its progression. The available evidence about AKT and/or PTEN in endometriosis supports the dysregulation of this pathway but refers to some *in vitro* and in vivo models [25–28] without taking into account the stage of the disease. Nevertheless, it is remarkable that typical signs and symptoms vary depending on the location and severity of the disorder [29].

Endometriosis could be poorly understood due to the limited accessibility to human tissue and limited handiness of menstruating animal models [30]. It becomes evident that many animal models used in preclinical studies do not recreate the real human conditions and are thus unsatisfactory. Therefore, in order to consider these issues, we focused on the complete patient biopsy.

Could the endometriosis stages be linked to differential changes in PI3K/AKT pathway regulation? From now on should we propose treatment strategies according each diagnosed stage? The aim of this work is to evaluate the expression of PI3K, PTEN, AKT and p-AKT in endometrial human biopsies, according to the presence or absence of the disease, and to assess the underlying differences regarding the endometriosis stages.

Materials and methods

Collection of biopsies

This study was approved by the Ethics and Research Committee from Instituto de Biología y Medicina Experimental, Ciudad Autónoma de Buenos Aires, Argentina.

A total of 29 female patients in reproductive age (mean age, 34 yr) who underwent diagnostic laparoscopy for infertility participated in this study: 20 with untreated endometriosis and 9 controls. Control subjects were women without endometriosis or any infectious or noninfectious pathology that could affect the evaluated biopsies, with tubal factor or unexplained infertility. All patients showed regular menstrual cycles and had not received any hormonal medical treatment for the last 6 months. All subjects signed informed consent prior to evaluation. Biopsies were obtained from all subjects in the proliferative phase, as described previously [31]. Biopsies of eutopic endometrium were taken with a metal Novak curette from the posterior uterine wall [32]. Ectopic endometrium biopsies belong to patients with peritoneal endometriosis. Further confirmation of the disease was performed by histological documentation. Lesions were classified according to the revised American Society for Reproductive Medicine (ASRM) classification of endometriosis [33]. From 20 patients with endometriosis, 11 were women with stages I-II and 9 were women with stages III–IV of the disease. The collected samples were immediately frozen at −70 °C for subsequent protein extraction and later Western Blotting.

Protein extraction

Eutopic endometrial tissue and peritoneal endometriotic lesions were placed on ice and dissected into small pieces (1mm^3) in a petri dish. Tissues were resuspended in a ratio 100 mg:1ml lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0,1%SDS and 10%glycerol) supplemented with protease inhibitor cocktail (P8340, Sigma-Aldrich Corporation, USA) and phosphatase inhibitors (25 mM sodium fluoride, 0.2 mM sodium orthovanadate and 10 mM b-glycerophosphate) and homogenized with an Ultra-Turrax homogenizer (IKA Werk, Germany) in 3–4 cycles of 10 s among 30–40 s intervals. Samples were centrifuged at 4 °C for 10 min at 10000 g and the resulting pellets were discarded. Protein concentration in the supernatant was measured by the Bradford assay.

Western blotting

Equal amounts of protein extracts (40 µg) were loaded into each lane of a 12% or 10% SDS-polyacrylamide gel and electrophoresis was performed at 150 V. The resolved proteins were transferred onto nitrocellulose membranes at 80 V and 4 °C for 2 h. The blot was then incubated in blocking buffer (5% nonfat milk, 0.1%Tween-20 in 20 mM TBS pH8.0) for 1 h at room temperature and incubated overnight at 4 °C with appropriate primary antibodies: 1/500 rabbit polyclonal anti-PI3K p110β (sc-602) and 1/400 rabbit polyclonal anti-phosphorylated Ser473 AKT (sc-7985-R) purchased from Santa Cruz Biotechnology (Santa Cruz, USA); 1/500 rabbit polyclonal anti-PTEN (07-1372) from Merck Millipore (Burlington, USA); 1/5000 rabbit polyclonal anti-AKT (#9272) and 1/10000 rabbit monoclonal anti-GAPDH (#2118) purchased from Cell Signaling Technology (Danvers, USA); and 1/2000 mouse monoclonal anti-β-actin (Ab6276) from Abcam (Cambridge, UK). Blot was then incubated with anti-mouse or anti-rabbit secondary antibodies conjugated with horseradish peroxidase and signal was detected by chemiluminescent substrate ECL (Pierce, Rockford, USA). The protein levels were analyzed by densitometry quantification using ImageJ 1.42q software (NIH) and expressed as arbitrary units.

Statistical analysis

Statistical analyses were performed using GraphPad PRISM Software V6.0 (GraphPad Software Inc., USA). Statistical comparisons between three different groups of biopsies were performed using parametric one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Differences between two different endometriosis stages were analyzed using Student's t-test. Results were expressed as mean ± SEM. In all cases, statistical significance was considered to be $p < .05$.

Results

PI3K/AKT pathway expression in endometrium from women with and without endometriosis

In order to assess the differences in PI3K/AKT pathway in the presence or absence of endometriosis we compared PTEN, PI3K, AKT and p-AKT protein levels in endometrial tissue from control women and endometriosis patients, as well as in endometriotic lesions (Figure 1). Eutopic and ectopic tissue from patients with endometriosis showed a significantly over-expression of PI3K in addition to higher levels of p-AKT/total AKT ratio. Eutopic and ectopic endometrium from patients with endometriosis showed a 97% and 85% increase of PI3K expression respectively compared to control endometrium ($p = .0004$ and $p = .002$ respectively). As well, eutopic and ectopic endometrium showed 163% and 256% higher levels of p-AKT/total AKT ratio respectively ($p = .0009$ and $p < .0001$ respectively) compared to the endometrium from control women (Figure 1(a,b)). Besides, both

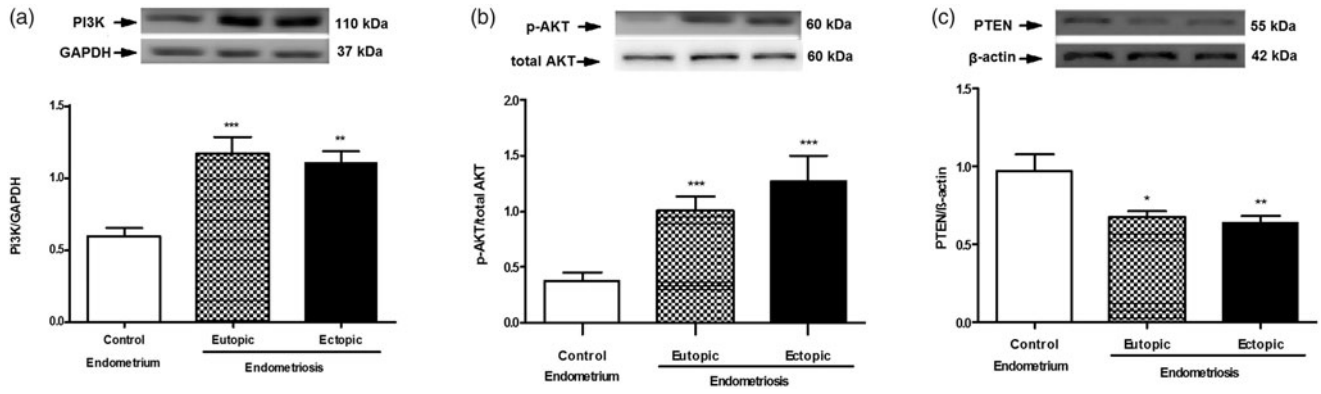

Figure 1. PI3K/AKT pathway expression in endometrium from women with and without endometriosis. The catalytic subunit of PI3K (a), p-AKT/total AKT ratio (b) and PTEN (c) protein levels were evaluated by western blot in endometrial tissue from control women and in eutopic and ectopic endometrium from patients with peritoneal endometriosis. GAPDH or β-actin was used as loading control. Both proteins from ratio p-AKT/total AKT were resolved in different gels and normalized to their own loading control band. Optical density is expressed as arbitrary units ± standard error. Representative immunoblots of protein content are shown in the upper panel. $^{*}p < .05$, $^{**}p < .01$ and $^{***}p < .001$ vs. Control Endometrium. Control: $n = 9$. Endometriosis: $n = 10$.

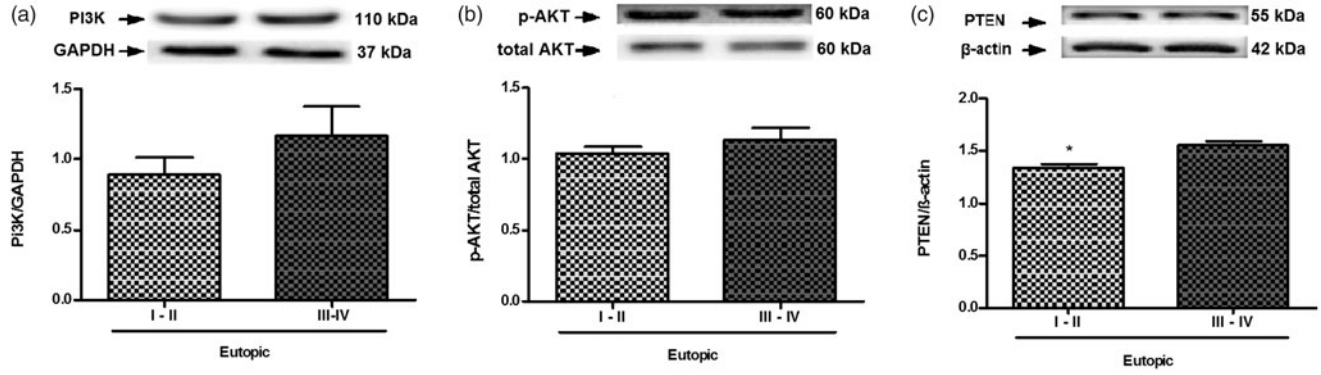

Figure 2. PI3K/AKT pathway expression in eutopic endometrium from patients with different stages of endometriosis. The catalytic subunit of PI3K (a), p-AKT/total AKT ratio (b) and PTEN (c) protein levels were evaluated by western blot in eutopic tissue from patients with minimal to mild endometriosis (stages I and II) and with moderate to severe endometriosis (stages III and IV). GAPDH or β-actin was used as loading control. Both proteins from ratio p-AKT/total AKT were resolved in different gels and normalized to their own loading control band. Optical density is expressed as arbitrary units ± standard error. Representative immunoblots of protein content are shown in the upper panel. $^{*}p < .05$. Eutopic I-II: $n = 5$. Eutopic III-IV: $n = 5$.

eutopic and ectopic endometrium from patients with endometriosis revealed an expression of PTEN 30% and 34% lower compared to control endometrium ($p = .049$ and $p = .0088$ respectively, Figure 1(c)).

PI3K/AKT pathway expression in eutopic and ectopic endometrium from patients with different stages of endometriosis

Since the observed differences in all these key proteins from PI3K/AKT pathway between control and endometriosis tissue, we wonder if these differences depended on the severity degree of the disease considering the diverse characteristics and symptoms from each stage. To approach this analysis we decided to group on one side the endometrial and endometriotic biopsies of patients with minimal to mild endometriosis (stage I and II), and on the other, the biopsies of patients with moderate to severe endometriosis (stage III and IV). Figure 2 shows the comparisons established in eutopic endometrial tissue between these two different groups of endometriosis stages and Figure 3 presents the ectopic endometrial tissue comparisons.

This study exposed no significant differences in the expression of PI3K between different stages of endometriosis neither in eutopic ($p = .5556$, Figure 2(a)) or ectopic ($p = .3898$, Figure 3(a)) endometrial tissue. However, both eutopic and ectopic endometrium from patients with minimal to mild endometriosis expressed 15% and 33% lower PTEN levels compared to the respective endometrium from patients with moderate to severe endometriosis ($p = .0286$ and $p = .0096$ respectively, Figure 2(c) and 3(c)). The ratio p-AKT/total AKT showed 144% higher levels of AKT phosphorylation in endometriotic tissue from patients with minimal to mild endometriosis compared to moderate to severe endometriosis ($p = .0412$, Figure 3(b)). These results suggest that the observed differences in PI3K expression in endometriosis patient respect to control women did not depend on the endometriosis stage. Otherwise, given that both tissues (eutopic and ectopic) showed a clear reduction in PTEN expression in patients with minimal to mild endometriosis, this could be one of the essential changes that could favors ectopic lesions development in the first stages of the disease. Even more, these changes in the pathway results in a marked increase of AKT phosphorylation in the ectopic lesions.

Discussion

Increased incidence of endometriosis and lack of effective therapeutic modalities urge a more in-depth understanding of the mechanisms and activated pathways in each patient that could influence the development and severity of the disease. As in any multifactorial and heterogeneous pathology the diversity of

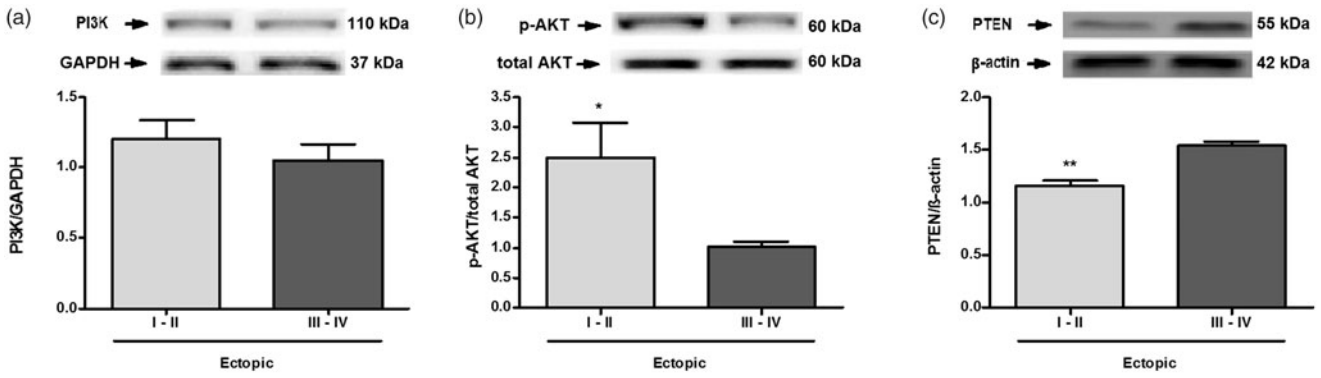

Figure 3. PI3K/AKT pathway expression in ectopic endometrium from patients with different stage of endometriosis. The catalytic subunit of PI3K (a), p-AKT/total AKT ratio (b) and PTEN (c) protein levels were evaluated by western blot in ectopic tissue from patients with minimal to mild endometriosis (stages I and II) and with moderate to severe endometriosis (stages III and IV). GAPDH or β-actin was used as loading control. Both proteins from ratio p-AKT/total AKT were resolved in different gels and normalized to their own loading control band. Optical density is expressed as arbitrary units ± standard error. Representative immunoblots of protein content are shown in the upper panel. $^{*}p < .05$, $^{**}p < .01$. Ectopic I-II: $n = 7$. Ectopic III-IV: $n = 8$.

symptoms and characteristics in each different diagnose, is an important point that should not be overlooked when investigating therapeutic strategies. Even more, it should be noted that it is not always a direct relationship between the degree of impairment and severity of symptoms [34].

Previous studies further explored the association between endometriosis and PI3K/AKT cascade, and compared levels of AKT phosphorylation in normal, eutopic and ectopic endometrium by immunohistochemistry among other in-vitro techniques [25,26]. However, the expression of this pathway in biopsies of different endometriosis stages remains unclear.

Our study revealed an increased expression of the catalytic subunit of PI3K in eutopic and ectopic endometrium from patients with endometriosis, as well as a reduced expression of PTEN and increased levels of AKT phosphorylation, compared to control endometrium. These results from peritoneal endometriosis are concomitant with previous studies where p-AKT and PTEN showed the same pattern in cell cultures performed from ovarian endometriomas [27]. All these together support the dysregulation of PI3K/AKT pathway in endometriosis and suggest that its treatment will not be successful by simply inhibiting estrogen production; the strategy must also disrupt the distorted PI3K/AKT signaling. Likewise, this pathway is one of the most frequently altered signaling pathways in endometrial cancer [35] and it has been deeply demonstrated that high activation of PI3K/AKT/mechanistic target of rapamycin kinase (mTOR) is essential for its progression [36]. Moreover, when inhibiting the phosphorylation of mTOR, there is a marked inhibitory effect on the proliferation of endometrial cancer [37]. Indeed, some PI3K/AKT inhibitors were recently shown to have therapeutic benefits through the prevention of endometriosis progression in vivo and *in vitro* [26,38]. Among others, Kacan et al. [39] considered to everolimus, an inhibitor of mTOR pathway, as possible treatment for endometriosis. In this recent study, the inhibition of mTOR significantly decreased endometriosis development and suppressed the endometriotic foci in an experimental model in rats. Nevertheless, by contrast to cancer therapy, the administration of PI3K/AKT pathway inhibitors in women with endometriosis appears quite controversial where the goal is improving the quality of life, rather than survival as primary endpoint. In fact, endometriosis needs long-term therapy combining prevention of implants recurrence, control of disease-related pain, acceptable costs and tolerability [40,41]. Given this background, the aim of future research should emphasize the feasibility of the proposed treatment.

In the present work, the altered expression of PI3K observed in the endometrium of patients with endometriosis, did not display significant changes due to the degree of severity. However in case of PTEN expression and AKT phosphorylation, we observed that both dysregulations in eutopic and ectopic tissue depended on the stage of the disease. It is known that at early stages of the endometriosis development the ectopic lesions are highly metabolically active [42]. The reduced expression of PTEN in endometriotic lesions from patients with minimal to mild degree of the disease, as well as the increased AKT phosphorylation, support this idea and highlight the importance of this pathway in the first stages of the disease. Moreover could validate these proteins as possible targets for future therapeutic strategies, maybe addressed to the high recurrence of the disease.

On the other hand, this is the first evidence of a reduced expression of PTEN in the eutopic endometrium from patients with minimal to mild degree of the disease, suggesting intrinsic changes in eutopic endometrium that could led to the lesion development in an ectopic site. Even more, it could be linked to the frequent recurrence of the disease, whereas it is not a particular variation of the ectopic tissue.

The understanding of the dynamic and progressive nature of eutopic and further ectopic endometrium help us to identify biomarkers for a future noninvasive diagnosis and the awareness of key pathophysiological mechanisms and potential targets [40]. The most interesting aspect of our finding is its contribution to knowing one of the possible causes why disease appears with different conditions in every patient. Keeping this in mind, further therapeutic strategies require to be developed according to the diagnosed stage of the disease, in order to suit the patient's need.

Disclosure statement

The authors declare that there is no conflict of interest.

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