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Differential Expression in Endometriosis Tissue versus Endometrium of the Uterine Adenogenesis Factors PRL-R, GH, IGF1, and IGF2

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ABSTRACT: Endometriosis is characterized by the presence of endometrial glandular and stromal structures outside the uterine cavity. It is an inflammatory estrogen dependent disease characterized by gene polymorphisms. This is a very frequent pathology and represents one of the most important causes of infertility, as well as having an important level of morbidity in patients. Recently, an alteration of the processes of organogenesis of the uterus has been proposed as a pathogenetic mechanism of endometriosis. In this article we have compared the expression in deep endometriotic lesions and in normal endometrial tissue of some of the molecular factors known to be involved in the embryonic development of the uterine glands. In detail, we found by immunohistochemistry a significant higher expression both for epithelium and stroma in the controls respect to the endometriosis samples for insulin growth factor 1 (IGF1) and IGF2, whereas for the prolactin receptor (PRL-R), this result was detected only for the epithelium. On the other hand, we found for growth hormone (GH) a significant higher expression in the epithelium of endometriosis samples respect to the controls. The correlation data generated can give indications on some of the molecular mechanisms responsible for the adenogenesis and survival of endometriosis structures outside of the uterus.

KEY WORDS: adenogenesis, endometriosis, endometrium, growth factors, uterus

I. INTRODUCTION

The basic structure, as well as the specific embryological and postnatal development stages of the uterus in mammals are quite well known. Nevertheless, there is a growing body of knowledge about genetic factors involved in uterine development and in potential role of early development in adult-onset diseases.[1] In all mammals, the complex of uterus, cervix, oviducts and anterior vagina develops as a specialization of the Mullerian ducts.[2] This is a complicated and highly regulated spatio-temporal mechanism of organogenesis, for which there is an articulated genetic network whose members are not yet fully known.[3] Alterations of this mechanism of organogenesis, the so-called anomalies of the Müllerian ducts, are relatively common.[4] In fact, the uterus is a very common site of developmental anomalies. The exact value of these anomalies is not known as many patients can remain asymptomatic for life. However, it is estimated that the prevalence ranges from 0.1 to 7% of the population and that this prevalence rises dramatically in patients with fertility and miscarriage problems.[5,6] It is interesting to note that this trend is perfectly superimposable to that recorded for endometriosis.[7,8] This disease is characterized by the presence of endometrial glandular and stromal structures outside the uterine cavity.[9] When endometriotic lesions are localized below the peritoneum, the term deep endometriosis is used.[10] To date, this is a very frequent pathology and represents one of the most important causes of infertility, as well as having an important level of morbidity in patients.[11,12] Despite this, the pathogenesis of endometriosis is not fully defined, the diagnosis is often late and there is no effective therapy for its elimination.[13] The theory still most accepted today for its pathogenesis is that of retrograde menstruation proposed by Sampson about a century ago. Other theories propose phenomena of metaplasia of coelomic tissue or activation of stem cells.[14] The presence of ectopic endometrium in fetal

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age has recently been demonstrated in the same anatomical compartments where endometriosis is normally found in adult patients.[15–17] This observation made it possible to define as one of the sure pathogenetic mechanisms of endometriosis, an alteration of the processes of organogenesis of the uterus and to include this disease in the complex of anomalies of the Müllerian ducts.[18] Indeed, the association between Mullerian duct anomalies and endometriosis is well known and has so far been interpreted in the context of the theory of retrograde menstruation.[8] In light of the data generated on the presence of ectopic endometrium in the fetal age, it is possible to hypothesize that endometriosis represents one of the phenotypes resulting from the organogenesis anomalies of the Müllerian ducts.[18] The molecular mechanisms responsible for this alteration of the fine regulation of uterus organogenesis are not known. Epithelial-mesenchymal interactions are crucial factors for a correct uterine development.[19] Indeed, these exchanges are responsible for local control and coordination of morphogenetical vital cell actions, such as cell movement and adhesion, cell differentiation and cell proliferation.[1] Furthermore, it must be underlined the role of hormonal input in developmental biology. In detail, more than ovarian hormonal input, seems to be important the role of other hormones such as prolactin and growth hormone.[1,20] In this article, we have compared the expression in deep endometriotic lesions and in normal endometrial tissue of some of the molecular factors known to be involved in the embryonic development of the uterine glands. As described by Gray et al, there is a plethora of factors involved in endometrial adenogenesis, including ovarian and pituitary hormones and several growth factors.[1] In this work, we analyzed by immunohistochemistry the expression of the prolactin receptor (PRL-R) and growth hormone (GH), as well as the expression of insulin growth factor 1 (IGF1) and IGF2. The correlation data generated can give indications on some of the molecular mechanisms responsible for the adenogenesis and survival of endometriosis structures outside of the uterus.

II. MATERIALS AND METHODS

A. Patients and Tissue Samples

Retrospective histopathological evaluation was executed on surgical specimens from patients who underwent surgery for infertility, pelvic pain symptoms or adnexal masses between 2000 and 2010 at the “Centro Italiano Endometriosi.” Only the samples where the occurrence of endometriotic glands was established histologically were designated in this study. After selection, 42 cases were eligible for the analysis. All patients provided written informed consent before enrollment in the study to permit the use of the data generated in retrospective analyses. The Human Normal Endometrium Tissue MicroArray CYN1 (SUPER BIO CHIPS, Seoul, Korea) including 59 normal endometrial samples both in the proliferative and secretive phase was used to define the expression of each antigen analyzed in the physiological endometrium. Main patients’ characteristics are summarized in Table 1. The study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2000 and 2008. The study was approved by the Scientific Committee of Fondazione Italiana Endometriosi.

TABLE 1: Characteristics of the 42 patients (age 35 ± 5 years) included in the study

	n	**%**
AFS stage*		
I	0	0
II	1	3
III	3	7
IV	38	90

*American Fertility Society stage based on The American Fertility Society, 1985.

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B. Immunohistochemistry

Immunohistochemistry was performed as previously described.[21,22] Briefly, 5-μm sections embedded in paraffin, were cut, mounted on glass and dried overnight at 37°C. All sections were then deparaffinized in xylene, rehydrated through a graded alcohol series and washed in phosphate-buffered saline (PBS). PBS was used for all subsequent washes and for antiserum dilution. Tissue sections were quenched sequentially in 3% hydrogen peroxide in aqueous solution and blocked with PBS-6% non-fat dry milk (Bio-Rad, Hercules, CA, USA) for 1 h at room temperature. Slides were then incubated at room temperature at 1:100 dilutions with the following antibodies: human GH antibody (ab74324), PRL-R (abx301880), IGF1 antibody (abx448548), and IGF 2 antibody (abx102887), all from Abcam. After three washes in PBS to remove the excess of antiserum, the slides were incubated with UltraTek HRP secondary antibody (ScyTek Laboratories, Logan, UT, USA) for 1 h. All the slides were then processed by the ABC method (Vector Laboratories) for 30 min at room temperature. Diaminobenzidine (ScyTek Laboratories) was used as the final chromogen and hematoxylin was used as the nuclear counterstain. Negative controls for each tissue section were prepared by leaving out the primary antiserum. As positive controls tissue samples expressing the antigen of interest were used. All samples were processed under the same conditions. The intensity of the different antigen expression was analyzed observing the number of positive cells and the strength of the expression and described as: score 1 (absent to very low); score 2 (low); score 3 (moderate), and score 4 (intense). An average of 22 fields was observed for each specimen. The level of concordance, expressed as the percentage of agreement between the observers was 92% (39 cases of 42). In the remaining specimens, the score was obtained after collegial revision and agreement.

C. Statistical Analyses

Descriptive analysis was performed by using median values and 95% confidence interval (CI) and percentages when appropriate. Differences between two groups of patients were assessed using Mann-Whitney *U* test for non-parametric continuous variables. Moreover, the different distribution of other variables between groups was assessed using chi-square test. SPSS software (version 17.00, SPSS, Chicago) was used for statistical analysis. $P < 0.05$ was considered to indicate statistical significance.

III. RESULTS

Stromal and epithelial endometriosis samples and normal epithelial and stromal endometrium controls were examined indifferently in the proliferative and secretory phases. Histologic examination of endometriosic lesions of the rectovaginal septum showed the distinctive presence of both endometriotic glands and stroma. The glands displayed endometriod characteristic, whereas the endometriotic stroma was similar to eutopic inactive or proliferative endometrial stroma. Moreover, commonly there was an infiltrate of histocytes with lipofuscin and hemosiderin pigment, possibly caused by hemorrhage and menstrual changes in endometriosis. Finally, the presence of a network of small arterioles, proliferation of nervous fibers and nodular aggregates of smooth muscle was commonly detected in the stroma.

We analyzed by immunohistochemistry the expression of PRL-R and GH hormones, as well as the expression of IGF1 and IGF2 in normal human endometrium and stroma and compared this expression with the one detected in the epithelium and stroma of a series of endometriosis samples. For all the antibodies tested, we detected a specific cytoplasmatic immunopositivity with no staining in the nucleus or at the level of the cytoplasmic membrane. The main characteristics of the cohorts analyzed are depicted in Table 1. In Figs. 1 and 2, the statistical analysis by means of Student's *t*-test of the differential expression of all these markers in epithelium and stroma of endometriosis lesions respect to normal human endometrium tissues is illustrated. We found a significant higher expression both for epithelium and stroma in the controls respect to the endometriosis samples for IGF1 and IGF2, whereas for PRL-R this result was detected only for the epithelium. Interestingly, we found for

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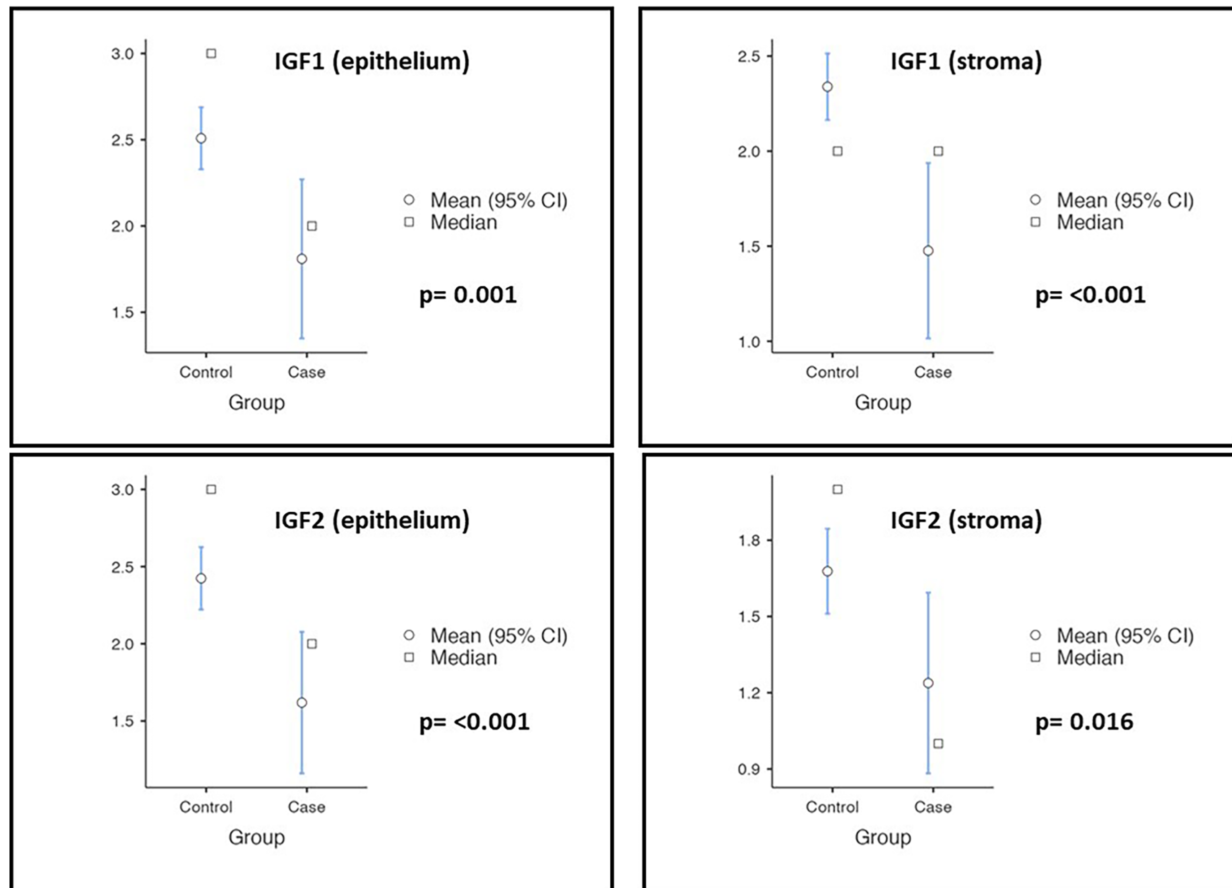

FIG. 1: Statistical analysis by means of Student's *t*-test of the differential expression of IGF1 and IGF2 in epithelium and stroma of endometriosis lesions with respect to normal human endometrium tissues

GH a significant higher expression in the epithelium of endometriosis samples respect to the controls. In Fig. 3, some exemplificative immunohistochemical staining are depicted.

Finally, to evaluate whether estrogen and progesterone levels could influence the expression of these endometrial adenogenesis factors in the uterus, we performed a statistical analysis of the immunohistochemical expression of these growth factors with respect to the phase of the hormonal cycle of the uterine tissue samples used as controls. Indeed, the statistical analysis highlighted differences in the immunohistochemical expression of GH (P = 0.001) and PRL-R (P = 0.024) in the epithelium in the two different phases of the cycle, proliferative and secretory, with a significant higher expression in the secretive phase. In endometriotic tissue this trend was not found for both GH and PRL-R (data not shown).

IV. DISCUSSION

Endometriosis is generally considered a gene polymorphism, inflammatory, estrogen-dependent disease However, the observation that this disease displays a high percentage of recurrence on patients treated with antiestrogens or GnRH analogs, suggests the possibility of different pathways for cell growth and differentiation.[23] Leaving from the observation that endometriosis could represent one of the phenotypes resulting from the organogenesis anomalies of the fetal uterus,[18] it would be interesting investigate the expression of proteins involved in embryogenesis of the uterus in endometriosis

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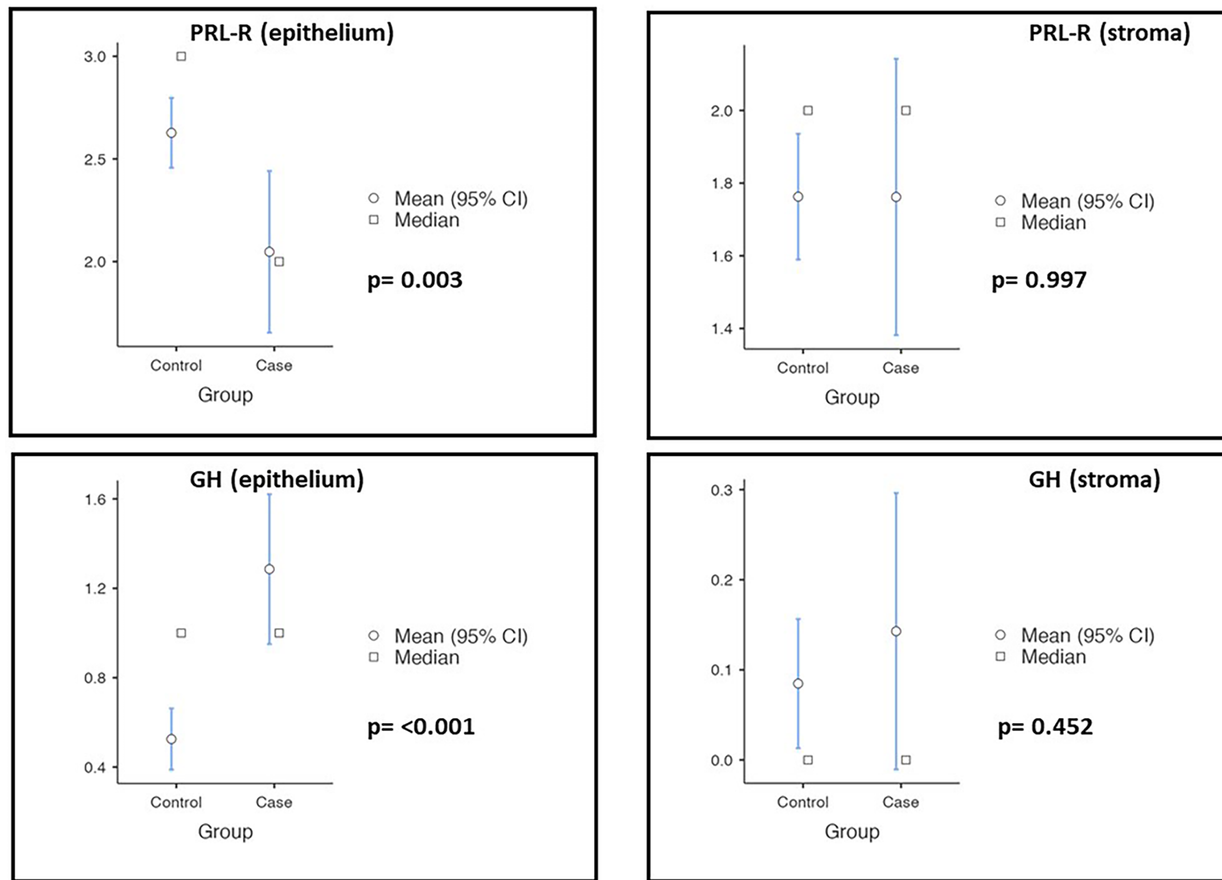

FIG. 2: Statistical analysis by means of Student's *t*-test of the differential expression of PRL-R and GH in epithelium and stroma of endometriosis lesions with respect to normal human endometrium tissues

lesions. Noteworthy, in the scientific literature there are only very few observations about the expression of these molecules in endometriosis in comparison to normal endometrium and no articles analyzing all of them together. On the other hand, it is well known that ectopic endometrial cells exhibit reduced spontaneous apoptosis respect to eutopic endometrial cells, and that the potential role of different growth factor must be investigated.[24] GH and PRL-R are synthesized and released from the pituitary gland into systemic circulation.[25] GH or PRL-R gene expression occurs also broadly in many of their central and peripheral sites of action, suggesting that they have a role in peripheral tissues ontogeny and homeostasis.[26] Several lines of evidence, indeed, suggest that alterations in this autocrine and/or paracrine roles are involved in both developmental and adult diseases.[25] It is well accepted that endometrial adenogenesis depends, at least in part, from a correct epithelial-mesenchymal interaction and that both PRL-R and GH hormones play a role in this by acting in autocrine/paracrine fashion, being IGF1 and IGF2 downstream targets involved in regulation of cellular growth and differentiation.[1,2,26,27] The expression of these last proteins in uterus, as well as in endometriosis is well documented.[1,27] It has been described at mRNA level that IGF is decreased in endometriotic cyst compared to endometrium of patients with endometriosis.[27] This result is in agreement with our data on IGF1 and IGF2 higher expression in normal endometrium respect to endometriosis lesions. Concerning GH, a single article showed a higher expression for GH in endometriosis.[28] In our experimental setting, we

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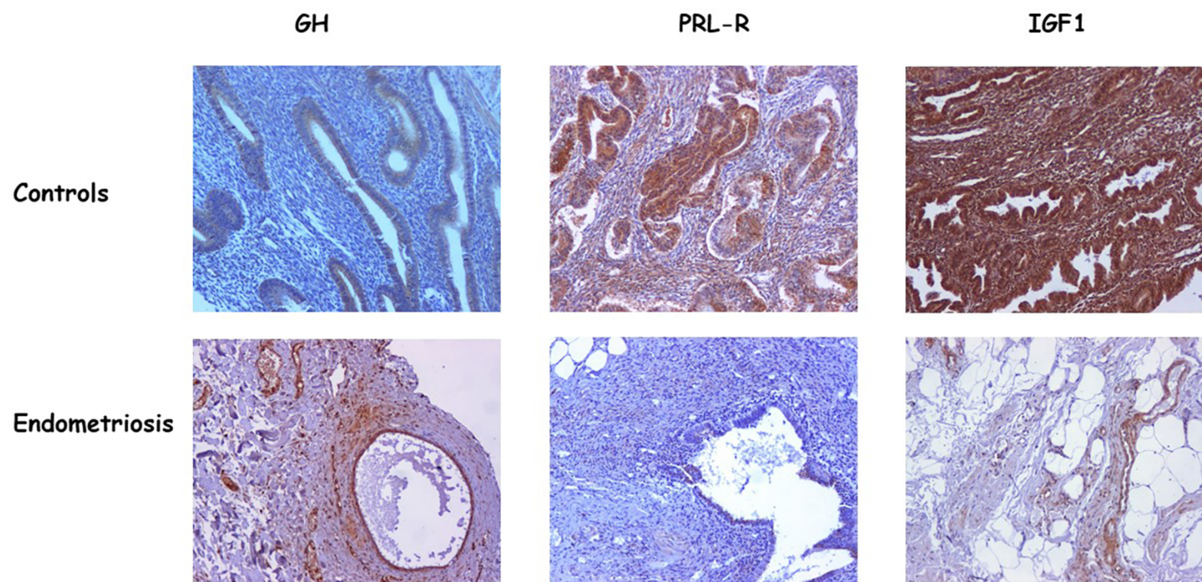

FIG. 3: Exemplificative immunostainings for GH, PRL-R, and IGF1 in human endometrium and endometriosis lesions displaying different level of expression (original magnification 10×).

have confirmed this significantly higher expression of GH in the epithelium of endometriosis structures respect to control endometrium. Considering the well-defined role of GH in promoting cellular proliferation and reducing cell-cell adhesion, it is possible to hypothesize that, during embryogenesis, this higher expression, could allow individual cells to break away from their parental architecture and could contribute to the formation of the primitive endometriosis lesions. Conversely, this altered expression could contribute to the progression of the disease in adult. Interestingly, we found an opposite expression pattern for PRL-R, as well as for IGF2 in our cohort of patients, whereas for IGF1, we did not find any significant difference. This observation is in accordance with data from the literature.[24–26] Interestingly, both PRL-R and IGF1 are clearly over-expressed respectively in the serum and in the peritoneal fluid of women with endometriosis.[26,29] The data produced in this article seems to suggest a role for PRL-R and IGF in the progression of endometriosis, but not in the pathogenesis of the disease. In fact, we have not been able to confirm at the tissue level the higher expression of these molecules in the serum and peritoneal fluid of the women of endometriosis. On the other hand, this extracellular over-expression could contribute to the creation of an environment favorable to the survival and growth of the endometrium outside the uterus.

Concerning the possible role of estrogen and progesterone levels on the expression of the factors analyzed in the article, we found, in accordance with data of the literature an increase in the expression of GH and PRL-R during the secretive phase of the human endometrium samples.[30] Conversely in the stromal and epithelial endometriotic tissues, GH and PRL-R expression was not linked to proliferative or secretory phase. This data suggests that some factors of adenogesesis in endometriotic tissue could display a regulation disengaged by the action of female sex hormones in the formation and sustenance of the disease. Functional *in vitro* and *in vivo* experimentations are required in order to eventually confirm this hypothesis.

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