Phosphatase of regenerating liver-3: a novel and promising marker in human endometriosis

Fei Ruan, M.D., Jun Lin, B.D., Rui-Jin Wu, Ph.D., Kai-Hong Xu, B.D., Xin-Mei Zhang, M.D., Cai-Yun Zhou, B.D., and Xiu-Feng Huang, M.D.

Department of Obstetrics and Gynecology, Women’s Hospital, School of Medicine, Zhejiang University, Hangzhou, People’s Republic of China

Objective: To investigate the expression of phosphatase of regenerating liver-3 (PRL-3) in ectopic, eutopic, and normal endometria and explore its relationship with endometriosis.

Design: A clinical retrospective and molecular study.

Setting: Department of obstetrics, gynecology, and reproduction medicine.

Patient(s): One hundred and five women with histopathologically confirmed endometriosis, and 50 women with histopathologically assessed normal endometria.

Intervention(s): Immunohistochemical staining and Western blot analysis.

Main Outcome Measure(s): Expression of PRL-3 protein.

Result(s): As shown by the immunohistochemical analysis, PRL-3 was mainly located in the cytoplasm and membrane. The cells that tested positive for PRL-3 were detected in endometriotic tissues that did not occur in eutopic and normal endometria. Statistical analysis indicated that the expression of PRL-3 was closely associated with the clinical stages and recurrence of endometriosis.

Conclusion(s): Expression of PRL-3 is related to the clinical stages and recurrence of endometriosis, which provides use with a novel marker and promising target in the treatment of human endometriosis. (Fertil Steril® 2010;94:1980–4. ©2010 by American Society for Reproductive Medicine.)

Key Words: Endometriosis, migration, phosphatase of regenerating liver-3

Endometriosis is a common benign gynecologic disorder affecting women’s physical and mental health, quality of life, and even fertility. The frequently used treatments in clinical practice are surgery and medication, but the recurrence rate is still as high as 20% to 50%. To date, no effective treatments have been found to reduce recurrence. Several theories, including Sampson’s theory of retrograde menstruation (1), have been proposed to explain the etiology of endometriosis, but the exact pathogenesis and related infertility remain unclear. For example, how do the endometrial cells migrate from the uterine cavity to the ectopic location—through a series of complex processes that include adhesion, invasion, and angiogenesis? Furthermore, how do these cells process the signals that result in migration, and what key regulators are required in this process? Revealing the mechanisms involved in the migration of endometriotic cells is imperative.

Phosphatase of regenerating liver-3 (PRL-3) has recently been a focus in cancer research as a promising prognostic factor and therapeutic target in metastatic tumors (2–5). In healthy adults, PRL-3 messenger RNA (mRNA) is primarily expressed in skeletal muscle and the heart, with relatively low expression levels in the pancreas, spleen, lung, and testis. No expression is found in the brain, liver, kidney, or placenta. Since Saha et al. (6) found PRL-3 was closely related to the metastasis of colon cancer, more studies have shown its occurrence in a variety of metastatic cancers. Zeng et al. (7) revealed that the overexpression of PRL-3 promoted the migration, invasion, and metastasis of cells in the Chinese hamster ovary (CHO). However, PRL-3 deletion induced by small interfering RNA (siRNA) was found to suppress the growth of ovarian cancer cells (8). Therefore, PRL-3 may play an important role in cancer metastasis and could act as a promising target in cancer therapy (9–11).

It is well known that cytoskeleton change, loss of adhesion, enhanced mobility, and degradation of basement membranes are involved in the metastasis of cancer cells. Meanwhile, studies have found that endometriosis shares a similar biological phenotype with malignant tumors in its excessive proliferation, metastasis, and invasion. So PRL-3 may be also involved in the migration, adhesion, and invasion of endometriotic cells. We investigated the expression of PRL-3 in endometriotic tissues and explored the relationship between PRL-3 and endometriosis in view of furthering our understanding of the pathogenesis, diagnosis, and treatment of endometriosis.

MATERIALS AND METHODS

Samples

Firstly, from 2004 to 2005, we obtained a total of 170 ectopic, eutopic, and normal endometrial samples from the Department of Pathology of the Women’s Hospital, Zhejiang University, China, for immunohistochemical staining. The ectopic (n = 70) and eutopic (n = 70) endometria were obtained from women with histopathologically confirmed endometriosis who had undergone laparoscopy or laparotomy. Among these women, 38 patients had a diagnosis of infertility, and the average time of infertility was 3.2 years (1 to 9 years). Endometriosis staging was defined according to the revised American Fertility Society classification system. Stage III (n = 30) and stage III/IV (n = 40) were regarded as early and late stage, respectively. Moreover, 30 patients (20 in the follicular phase, and 10 in the secretory phase) without
endometriosis were recruited from our hospital as controls. After the operation, the 70 patients with endometriosis were followed up for 3 years; during this period, in the 38 infertile women, 20 had endometriosis recurrence and 17 had pregnancies among 38 infertile women.

Additionally, in 2008 we recruited 35 patients with histopathologically confirmed endometriosis and 20 with histopathologically confirmed normal endometria for a Western blot analysis study. The patients with endometriosis underwent laparoscopy or laparotomy, and those without endometriosis received hysterectomy for other benign gynecologic disease including uterine fibroids. The samples from these patients were stored at –80°C until use.

All patients had regular menstrual cycles, and their last menstrual memory was accurate. None had received any hormone therapy or used an intrauterine device during the previous 3 months. No other concomitant diseases were found. The mean age at the time of diagnosis was 35 years (range: 19 to 53 years) with no significant differences in demographic data between the groups. The study was approved by the institutional review board of our hospital, and there were no conflicts of interest.

Immunohistochemical Staining

Immunohistochemical staining was performed to detect the expression of PRL-3 in 170 samples. All samples were embedded in paraffin and cut into 5-μm sections. After baking at 60°C overnight, sections were deparaffined and rehydrated, then incubated with antigenic retrieval buffer containing ethylene diamine tetraacetic acid (EDTA, pH 8.0) and microwaved. The endogenous peroxidase activity was quenched by incubation of the sections in 3% hydrogen peroxide for 10 minutes at room temperature. Then the sections were treated with the nonimmune serum originated secondary antibody at room temperature for 30 minutes to reduce nonspecific binding. Subsequently, the sections were incubated with primary antibody to PRL-3 (1:600; Sigma, St. Louis, MO) overnight at 4°C, followed by incubation with the secondary antibody (Invitrogen, Carlsbad, CA) for 30 minutes at room temperature. After they were colored by the diaminobenzidine (DAB; Sigma), the sections were analyzed under a microscope. We selected the known positive paraffin section as a positive control and the phosphate-buffered saline (PBS) solution substituted for the primary antibody as a negative control.

Assessment of Staining

The PRL-3 was located in the cytoplasm and membrane and was stramineous, buffy, and brown. Five different fields (magnification ×400) were randomly selected in each section, and the number of cells that tested positive for PRL-3 was counted. The staining degree was scored as 0 (≤5%), 1 (6% to 25%), 2 (26% to 50%), and 3 (>50%) according to the percentage of cells positive for PRL-3. The staining intensity was scored as 0 (negative), 1 (stramineous), 2 (buffy), and 3 (brown). The products of the intensity and degree scores were used as the final scores (0 to 9) for PRL-3. We regarded 0 to 1 as negative (−), 2 to 3 as weakly positive (+), 4 to 6 as moderately positive (++), and ≥7 as strongly positive (+++). A final score ≥2 was regarded as positive. The assessment of immunohistochemical staining was performed independently by two experienced pathologists who were blinded to the study.

Western Blot Analysis

The samples were weighed, homogenized, and centrifuged at 14,000 rpm for 10 minutes at 40°C, and the supernatant was transferred to a fresh tube. Cell were lysed in 50 mmol/L Tris-HCl (pH 7.4), 250 mmol/L NaCl, 0.1% NonidetNP40, 5 mmol/L EDTA, and 50 mmol/L NaF in the presence of protease inhibitors for 30 minutes on ice. The protein concentration of the supernatant...
was determined using a Bio-Rad assay kit (Bio-Rad, Milan, Italy). The proteins were isolated electrophoretically in 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was dried at room temperature and blocked in 5% bovine serum albumin (0.25 g BSA in 5 mL of PBS-T) overnight under continuous shaking at 80 rpm. Then the membrane was treated with goat polyclonal antibody to PRL-3 (1:400; Santa Cruz Biotechnology, Santa Cruz, CA). The expression of PRL-3 was determined with horseradish peroxidase-conjugated anti-goat immunoglobulin G (Santa Cruz Biotechnology) and enhanced chemiluminescence (ECL; Sigma). Rabbit polyclonal antibody to β-actin (Invitrogen) was used as the internal reference.

**Statistical Analysis**

All statistical analyses were performed using the SPSS 12.0 statistical software (SPSS, Inc., Chicago, IL). The relationship between PRL-3 and clinical characteristics was analyzed with the chi-square test. P < .05 was considered statistically significant.

**RESULTS**

**Immunohistochemistry**

Immunohistochemical analysis showed that PRL-3 was mainly located in the cytoplasm and membrane of glandular epithelium cells, stromal fibroblasts, and vascular endothelial cells (Fig. 1). Expression of PRL-3 was observed in 72.9% (51 out of 70) of ectopic endometria samples. However, PRL-3 staining was either absent or only present in a few cells in the eutopic and normal endometria (Fig. 2). Statistical analysis indicated that PRL-3 expression in ectopic endometria was higher than in eutopic and normal endometria (P < .001). Moreover, the positive rate of PRL-3 expression was 53.3% (16 out of 30) and 87.5% (35 out of 40) for stage I/II and stage III/IV, respectively (P < .01). The results indicated that PRL-3 was expressed in 18 out of 20 women with recurrence, which was higher than that in women without recurrence (P < .05), suggesting that PRL-3 might be closely related with the occurrence, clinical stages, and recurrence of endometriosis. However, no relationship was found between endometriosis and age, sterility time, or pain (Table 1).

Moreover, there was no significant difference between patients with follicular phase and secretory phase ectopic endometria. As for glandular epithelium cells and stromal cells, the expression of PRL-3 was higher in the endometriotic stromal cells than in the

**TABLE 1**

Association of phosphatase of regenerating liver-3 (PRL-3) expression and clinical characteristics.

<table>
<thead>
<tr>
<th>Clinical characteristics</th>
<th>Cases (n)</th>
<th>Positive</th>
<th>Negative</th>
<th>Chi-square</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>38</td>
<td>24</td>
<td>14</td>
<td>0.10</td>
<td>NS</td>
</tr>
<tr>
<td>&gt;35</td>
<td>32</td>
<td>19</td>
<td>13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sterility time</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 years</td>
<td>16</td>
<td>11</td>
<td>5</td>
<td>0.07</td>
<td>NS</td>
</tr>
<tr>
<td>&gt;3 years</td>
<td>22</td>
<td>16</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pain</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>47</td>
<td>33</td>
<td>14</td>
<td>0.18</td>
<td>NS</td>
</tr>
<tr>
<td>No</td>
<td>23</td>
<td>15</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clinical stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I/II</td>
<td>30</td>
<td>16</td>
<td>14</td>
<td>10.12</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>III/IV</td>
<td>40</td>
<td>35</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pregnancy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>17</td>
<td>9</td>
<td>8</td>
<td>0.31</td>
<td>NS</td>
</tr>
<tr>
<td>No</td>
<td>21</td>
<td>13</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recurrence</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>20</td>
<td>18</td>
<td>2</td>
<td>4.16</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>No</td>
<td>50</td>
<td>33</td>
<td>17</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: NS, not statistically significant.
endometriotic glandular epithelium cells of patients with recurrence while no significant difference was observed between other groups.

**Western Blot Analysis**

The expression of PRL-3 was observed in 29 ectopic endometria, and the positive rate was 82.9% (29 out of 35). The gray value normalized to β-actin was 0.61 ± 0.18. However, PRL-3 expression was not detected in eutopic or normal endometria (Fig. 3) \( (P < .001) \).

**DISCUSSION**

Reversible protein tyrosine phosphorylation is a central mechanism in cellular signaling, leading to proliferation, migration, differentiation, motility, and adhesion. Tyrosine phosphorylation and dephosphorylation is balanced by a family of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). A member of the PTPs group, PRL-3 has recently been identified as a marker for metastasis of malignant tumors (12, 13). Through activating a series of intracellular signaling pathways, PRL-3 induces cell differentiation, proliferation, invasion, and metastasis (14–16). However, there are as yet no reports on the role of PRL-3 in the pathogenesis of endometriosis.

Endometriosis is a common benign disease that is characterized by the ectopic growth of endometrial cells, with a similar biological phenotype to malignant tumors in their excessive proliferation, invasion, and metastasis. As immunohistochemical analysis showed, PRL-3 was detected in 72.9% of endometriotic tissues, which was higher than that in eutopic and normal endometria. Consequently, we confirmed these findings with Western blot analysis, which revealed a 82.9% positive rate in endometriotic tissues. Furthermore, the positive rate of PRL-3 expression was 53.3% and 87.5% for stage I/II and stage III/IV, respectively \( (P < .01) \); this indicated that PRL-3 might be correlated with the clinical stages of endometriosis. In women with recurrence, the positive rate of PRL-3 (18 out of 20) was higher than in women without recurrence \( (P < .05) \). These results confirmed that PRL-3 has a close relationship with endometriosis and is associated with its clinical stages and recurrence.

In our study, the PRL-3 was mainly detected in the cytoplasm and membrane of glandular epithelium cells as well as in stromal cells such as fibroblasts and vascular endothelial cells. It is interesting that the expression of PRL-3 in endometriotic stromal cells was higher than their expression in endometriotic glandular epithelium cells among the patients with recurrence. Studies with a larger sample size are still needed to determine whether endometriotic stromal cells play a more important role than endometriotic glandular epithelium cells in the recurrence of endometriosis. Endogenous PRL-3 is linked with the plasma membrane and endosomal structures in a prenylation-dependent manner. Some studies have found the expression of membrane-associated PRL-3 induced dephosphorylation of substrates in the cell membranes to modulate the organization of plasma membrane in such a way to promote cell motility and invasion. Therefore, PRL-3 may be a promising marker in the diagnosis and prognosis of endometriosis.

It is well known that cell migration involves the formation of a pseudo-foot, adhesion of a new establishment, contraction of the cell body, and separation of the cell tail. However, the specific mechanism of migration of the endometriotic cells induced by PRL-3 was still unclear. The Ras homology (Rho) family of small guanosine triphosphatase (GTPase) was known as a “molecular switch” in the regulation of cell shape, motility, cell-substratum adhesion, and cell contraction by reorganizing actin cytoskeletons. Fiordalisi et al. (17) found that PRL-3 induced tumor metastasis by regulating the Rho family through the Rho/Rho kinase (ROCK) pathway. Yuge et al. (18) observed the overexpression of RhoA and ROCK in endometriotic stromal cells. In our study, the expression of PRL-3 was richly detected in endometriotic tissues, which indicated the close relationship between PRL-3 and endometriosis. These findings suggest that PRL-3 might induce migration of endometrial cell by activating the RhoA/ROCK signaling pathway. However, intracellular and extracellular signaling systems are complex, and a signaling network is formed through multilevel interactions with other signaling pathways. The exact biological function and cellular substrates of PRL-3 and the cellular signaling pathways mediated by PRL-3 have yet to be identified (19). It was hypothesized that PRL-3 might act as an upstream signal leading to cell proliferation and migration by activating a number of downstream signaling pathways (20, 21). Therefore, RhoA/ROCK signaling induced by PRL-3 may be not the only pathway involved in endometriosis.

Our study suggested that PRL-3 had a close relationship with the clinical stages and recurrence of endometriosis, which implied the clinical importance of PRL-3 in endometriosis. Furthermore, we postulated that the role of PRL-3 in endometriosis was mediated through activating certain signaling pathways to induce endometrial cell migration. Therefore, PRL-3 may be a promising marker and a potentially attractive target in endometriosis. Further studies are needed to investigate the specific mechanism responsible for the migration of endometrial cells induced by PRL-3 to reveal the pathogenesis of endometriosis and to explore a new therapeutic strategy.

**Acknowledgments:** The authors thank our colleagues for their technical assistance and helpful comments.


