Estrogen and progesterone receptors in ovarian epithelial tumors

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Received 12 February 2004; received in revised form 12 February 2004; accepted 17 February 2004

Abstract

Epidemiological studies have indicated a relationship between ovarian cancer and gonadal steroid hormones. In the present study immunohistochemical localization in combination with morphometry were used to characterize changes in the pattern of expression for estrogen receptor alpha (ERα), estrogen receptor beta (ERβ), and progesterone receptor (PR), in epithelial cells of normal ovaries, and in benign, borderline and malignant ovarian tumors of different types (n = 53). Positive correlations with immunoreactivity of the cell proliferation-marker, Ki67, and the apoptosis-related marker of genetic instability, p53, between the different tumor types were also found. A simultaneous expression of ERα/H9251, ERβ/H9252 and PR in epithelial cells of all histopathological tumor types was noted, with the notable exception of all mucinous tumors who remained ERβ-positive, but ERα- and PR-negative. Epithelial cells in ovarian cancer tissue showed significantly lower mean immunoreactivity of ERβ and PR, but not ERα, than in normal ovarian tissue. These novel findings may provide a rationale for the development of new diagnostic and possibly therapeutic strategies.

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Keywords: Steroid hormone receptors; Ki67; p53; Immunohistochemistry; Ovarian cancer; Ovarian tumors

1. Introduction

Ovarian cancer is the gynecological cancer with the highest mortality rate in western countries (Parker et al., 1997). A putative direct action of gonadal steroids in ovarian carcinogenesis has been suggested (Clinton and Hua, 1997; Risch, 1998; Emons and Kavanagh, 1999), which is supported by the findings of mRNA transcripts and translated proteins of progesterone receptor (PR) and estrogen receptors (ER) in both normal ovarian tissue and in malignant ovarian tumors (Rao and Slotman, 1991; Clinton and Hua, 1997; Lau et al., 1999a; Munstedt et al., 2000). While the progestrone receptor isoforms are derived from a single gene (Kastner et al., 1990) the known estrogen receptor variants are coded from two separate genes, ERα and ERβ, located on chromosomes 6q25.1 and 14q22-24, respectively (Greene et al., 1986; Enmark et al., 1997).

ERβ shares considerable homology in the DNA binding region (97%) with ERα, while this homology is markedly lower (55%) in the ligand-binding domain, which may explain the lower binding affinity for 17β-estradiol (Enmark and Gustafsson, 1999). The trans-activating mode of action of ERβ is similar to ERα (Petterson et al., 1997) and anti-estrogens can inhibit this effect (Mosselman et al., 1996; Tremblay et al., 1997). Moreover, ERβ and ERα are usually, but not always, co-expressed in many human tissues (Enmark et al., 1997; Taylor and Al-Azzawi, 2000). Although limited information is available regarding the role of ERβ in ovarian epithelial cancer (Lau et al., 1999b; Brandenberger et al., 1998; Pujol et al., 1998; Fujimura et al., 2001), previous studies have shown that ERα and PR are commonly found in more than 50% of carcinomas, for review see (Rao and Slotman, 1991; Clinton and Hua, 1997;
Miller and Langdon, 1997; Emmons and Kavanagh, 1999). However, their significance in relation to the development of ovarian cancer tumors and patient prognosis is still unclear. With the incentive to search for differences between various ovarian tumor groups, we used immunohistochemical methods to characterize the pattern of expression for ERα, ERβ, and PR in epithelial cells of normal ovaries and in benign, borderline and malignant tumors of different epithelial types. This pattern was related to Ki67 index and p53, which are established markers of proliferation (Gerdes et al., 1984) and apoptosis-related genetic instability (Steele et al., 1998), respectively.

2. Materials and methods

2.1. Patients

This study was approved by the Human Ethics Committee at the Medical Faculty of Umeå University and Umeå University Hospital. During the years 1993–1999, 53 women with suspected ovarian pathology were recruited, following informed consent. Upon laparotomy at the Department of Obstetrics and Gynecology and Gynecologic Oncology, Umeå University Hospital, the ovarian tumor(s) were removed from 45 women. Macroscopically and histopathologically normal ovaries, similarly removed for clinical reasons, were obtained from eight women. No patient had received chemotherapy. The mean age ± S.E. was 57.8 (1.9) years. As judged by patient history and hormone analyses, 15 women (28%) were pre-menopausal and 38 women (72%) post-menopausal at the time of surgery. The ovarian tumors were all WHO classified (Scully and Sobin, 1999) as surface epithelial-stromal (Table 1). Fifteen (33%) were judged malignant, 14 (31%) borderline and 16 (36%) benign (Table 1). Five (33%) of the ovarian cancers were highly differentiated (grade 1), three (20%) moderately (grade 2), and seven (47%) poorly differentiated (grade 3). Staging was performed according to FIGO. Eight patients (53%) were in stage I and seven (47%) in stage III.

Immediately after surgical removal, the ovary or ovarian tumor was transported to the Department of Pathology and dissected. Tumor tissue samples were fixed by immersion in sodium phosphate-buffered neutral 10% formalin. After 1–2 days of fixation, selected tissue blocks were routinely processed and paraffin embedded. Four micrometers thick sections were mounted on poly-l-lysine-coated slides (Sigma, St. Louis, MO). The sections were then deparaffinized, rehydrated and to quench endogenous peroxidase activity, incubated for 30 min with 3% H2O2 in methanol. After a short rinse in Tris buffer the sections were boiled in a microwave oven (Electro Helios, HC 749, set at maximal effect of 800 W) for 3 × 5 min in citrate buffer (Shi et al., 1991). Following cooling and rinsing in Tris-buffered saline, normal horse serum (for ERα, Ki67 and p53 antibodies), goat serum (for PR antibodies and ERβ14-A antibody) or rabbit serum (for ERβ503 antibody) was applied to the sections for 20 min in order to block non-specific binding. The sections were then incubated overnight at 4 °C with specific antibodies directed against ERα, ERβ or PR (recognizing both PR-A and PR-B isoforms), Ki67, and p53. Localization of antigen–antibody complexes was performed with the avidin–biotin–peroxidase complex (ABC) technique (Hsu et al., 1981) using a Vectastain ABC Kit. Peroxidase activity was demonstrated by 5 min incubation in 3,3′-diaminobenzidine tetrahydrochloride and H2O2 dissolved in citrate buffer. After rinsing the sections were counterstained with Mayer’s hematoxylin.

The following antibodies were used: ERα (ID5, DAKO A/S, Glostrup, Denmark and NCL-ER6F11, Novoceastra Laboratories Ltd., Newcastle-upon-Tyne, UK); PR (PgR-ICA, Abbott Laboratories, Abbott Park, IL, USA and NCL-PGR, Novocastra); Ki67 (MIB 1, Immunotech S.A., Marseille Cedex, France); p53, staining both wild and mutant types (p53Ab-6, Oncogene Science Inc., Uniondale, NY, USA); ERβ (polyclonal rabbit ERβ 14-A, ADI Inc., San Antonio, TX, USA and chicken ERβ503 IgY, KaroBio AB, Huddinge, Sweden). The preparation of a chicken polyclonal ERβ503 IgY has been described earlier in detail (Saji et al., 2000). Controls were performed according to the manufacturer’s recommendations. For each set of incubations internal positive and negative controls were present. The immunohistochemical specificity of ERβ was checked by pre-incubation with buffer and control immunogenic peptides: ERβ14-P (ADI Inc., USA) was used for ERβ14-A antibody, and recombinant human ERβ purchased from PanVera Corp (Madison, WI, USA) and pre-absorbed ERβ503 protein (KaroBio AB, Huddinge Stockholm) were used as antigen for the ERβ503 antibody.

The immunohistochemical staining was judged by two observers (SC, PL) using a standard light microscope. Each sample was evaluated regarding staining of epithelial cells

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Number (pre-/post-menopausal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal ovaries</td>
<td>8 (4/4)</td>
</tr>
<tr>
<td>Serous benign tumor</td>
<td>6 (2/4)</td>
</tr>
<tr>
<td>Serous borderline tumor</td>
<td>5 (4/1)</td>
</tr>
<tr>
<td>Serous cancer</td>
<td>5 (6/0)</td>
</tr>
<tr>
<td>Mucinous benign tumor</td>
<td>10 (6/4)</td>
</tr>
<tr>
<td>Mucinous borderline tumor</td>
<td>9 (2/7)</td>
</tr>
<tr>
<td>Mucinous cancer</td>
<td>5 (6/0)</td>
</tr>
<tr>
<td>Endometrioid cancer</td>
<td>5 (2/3)</td>
</tr>
<tr>
<td>Total</td>
<td>53 (15/38)</td>
</tr>
</tbody>
</table>
and staining of stromal tumor cells was thus not registered. In normal ovaries only ovarian surface epithelial cells were evaluated. Cells were considered positive only if nuclear staining was present. A Weibel 1 graticula (Graticules Ltd., Tonbridge, UK) was mounted in the eyepiece of the microscope and used according to stereological principles (Weibel, 1979). The percentage of positive cells was determined by counting stained and unstained cells crossed by the grid lines in 5–20 high-power fields (HPF, 40 × lens). One hundred cells were counted.

Nuclear staining of ERα, ERβ, and PR were measured. All immunohistochemistry was performed using two different antibodies. Similar immunostaining results were obtained between the different antibodies (ERα: ID5 versus NCL-ER-6F11, PR: PgR-ICA versus NCL-PGR and ERβ: ERβ 14-A versus ERβ 503). All showed a mixture of clearly positive and negative cell nuclei within each HPF. Both ERβ antibodies displayed a cytoplasmic staining besides the nuclear staining. Since ERβ 503 antibody showed no or only a weak cytoplasmic staining as compared with the more pronounced cytoplasmic staining of ERβ 14-A (data not shown), results presented herein are based exclusively on the ERβ 503 antibody (Wang et al., 2001).

The immunoreactivity of receptor-positive samples was semi-quantitatively assessed by two different methods. Results presented are based on a semi-quantitative estimation incorporating both frequency of stained cells and staining intensity classified on a scale: 0: no staining, 1: weak, 2: moderate, 3: strong staining (Lindgren et al., 2001). The precision of this scoring was assessed against the more commonly used immunoreactive score (IRS), described by Remmele and Stegner (Remmele and Stegner, 1987) and a very high degree of correlation was found (r_s = 0.87, P < 0.001, SRC).

Any nuclear staining of Ki67 was considered positive and no grading of staining intensity was performed. A clearly positive ERα expression was found in all cases of normal, benign, borderline and malignant ovarian tissues. Nuclear immunostaining was observed in ovarian epithelial cells as well as in stromal cells and vascular endothelium (Fig. 1). Immunoreactivity in epithelial cells of normal ovaries was higher than in benign, borderline and malignant tumors, P = 0.001, KW (Fig. 2A). When comparing different histopathological types, ERα staining intensity in epithelial cells of normal ovaries was significantly higher than in all other groups with the single exception of serous borderline tumors (Table 2). When benign, borderline and malignant tumors of different histological types were grouped together, a significantly lower mean immunoreactivity was found in mucinous tumors as compared with serous tumors (0.9 ± 0.1, n = 24 and 1.8 ± 0.1, n = 16, P < 0.001, MWU).

ERα immunoreactivity was found in all normal and tumorous ovaries with the notable exception of all mucinous

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>ERα immunoreactivity</th>
<th>Difference vs. normal ovaries (P-value)</th>
<th>ERβ immunoreactivity</th>
<th>Difference vs. normal ovaries (P-value)</th>
<th>PR immunoreactivity</th>
<th>Difference vs. normal ovaries (P-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal ovaries</td>
<td>2.00 ± 0.30</td>
<td>2.38 ± 0.13</td>
<td>2.00 ± 0.15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serous benign</td>
<td>1.42 ± 0.27</td>
<td>ns</td>
<td>1.92 ± 0.08</td>
<td>0.02</td>
<td>1.93 ± 0.46</td>
<td>ns</td>
</tr>
<tr>
<td>Serous borderline</td>
<td>2.00 ± 0.16</td>
<td>ns</td>
<td>1.9 ± 0.29</td>
<td>0.50</td>
<td>2.10 ± 0.20</td>
<td>ns</td>
</tr>
<tr>
<td>Serous cancer</td>
<td>1.50 ± 0.50</td>
<td>1.40 ± 0.19</td>
<td>&lt;0.01</td>
<td>0.30 ± 0.12</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td>Mucinous benign</td>
<td>10 nd</td>
<td>&lt;0.001</td>
<td>0.60 ± 0.10</td>
<td>&lt;0.001</td>
<td>nd</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mucinous borderline</td>
<td>9 nd</td>
<td>&lt;0.001</td>
<td>1.06 ± 0.13</td>
<td>&lt;0.001</td>
<td>nd</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mucinous cancer</td>
<td>5 nd</td>
<td>1.00 ± 0.16</td>
<td>&lt;0.01</td>
<td>0.001</td>
<td>nd</td>
<td>0.001</td>
</tr>
<tr>
<td>Endometrioid cancer</td>
<td>5 1.50 ± 0.22</td>
<td>ns</td>
<td>1.00 ± 0.27</td>
<td>0.001</td>
<td>1.10 ± 0.40</td>
<td>ns</td>
</tr>
</tbody>
</table>

All mucinous tumors were ERα- and PR-negative. ERα, ERβ, and PR immunoreactivity were significantly different between different tumor groups (Kruskal–Wallis test, P < 0.001). Mann–Whitney U-test was used to test the differences in immunoreactivity between normal ovaries and ovarian tumor groups. ns: not statistically significant, nd: not detectable.

3. Results

3.1. Expression of gonadal steroid receptors in normal and ovarian epithelial tumor cells

A clearly positive ERα expression was found in all cases of normal, benign, borderline and malignant ovarian tissues. Nuclear immunostaining was observed in ovarian epithelial cells as well as in stromal cells and vascular endothelium (Fig. 1). Immunoreactivity in epithelial cells of normal ovaries was higher than in benign, borderline and malignant tumors, P = 0.001, KW (Fig. 2A). When comparing different histopathological types, ERα staining intensity in epithelial cells of normal ovaries was significantly higher than in all other groups with the single exception of serous borderline tumors (Table 2). When benign, borderline and malignant tumors of different histological types were grouped together, a significantly lower mean immunoreactivity was found in mucinous tumors as compared with serous tumors (0.9 ± 0.1, n = 24 and 1.8 ± 0.1, n = 16, P < 0.001, MWU).

ERα immunoreactivity was found in all normal and tumorous ovaries with the notable exception of all mucinous
tumors (Fig. 3) and a single serous cancer. Mean ERα immunoreactivity in epithelial cells was significantly higher in normal ovaries than in benign, borderline and malignant ovarian tumors, $P = 0.01$, KW (Fig. 2A). However, after exclusion of the mucinous tumors ($n = 24$), which were all ERα-negative, the small difference in mean values of immunoreactivity was not significant, $P = 0.30$, KW (Fig. 2B).

PR showed a positive staining in all cases except in one endometrioid cancer, one benign and two malignant serous ovarian tumors, and as for ERα, all mucinous tumors were PR-negative (similar pattern as ERα illustrated in Fig. 3, data not shown). Mean PR immunoreactivity was significantly higher in normal ovaries than in benign, borderline and malignant ovarian tumors, $P < 0.01$, KW (Fig. 2A).

After exclusion of mucinous tumors, no clear differences were noted between normal ovaries and benign or borderline ovarian tumors. However, mean PR immunoreactivity remained significantly lower in ovarian cancer tumors as compared with normal ovaries ($0.7 \pm 0.2$ versus $2.1 \pm 0.1$, $P < 0.01$, MWU, Fig. 2B).

ERα, ERβ and PR were uniformly co-expressed in all tissues, with the notable exception of ERα and PR-negativity in mucinous tumors, irrespective of tumor type. The immunoreactivity of all steroid receptors measured in the entire material was significantly correlated: ERα and ERβ ($r_s = 0.64$, $P < 0.001$, SRC), ERα and PR ($r_s = 0.68$, $P < 0.001$), and ERα and PR ($r_s = 0.84$, $P < 0.001$). ERα, ERβ and PR immunoreactivity levels were not significantly different between pre- and post-menopausal women (data not
Panel (B), all mucinous tumors excluded (n = 1). When calculating the ratio of ER and PR, statistical differences between normal ovaries and ovarian tumor groups by Kruskal–Wallis test and Mann–Whitney U-test as a post hoc, *P < 0.05, **P < 0.01, ***P < 0.001. Panel (A), all cases (n = 53). Panel (B), all mucinous tumors excluded (n = 29).

Fig. 2. Immunoreactivity indices (mean ± S.E.) of ERα, ERβ, PR in epithelial cells of normal ovaries, benign, borderline and malignant ovarian tumors. Statistical differences between normal ovaries and ovarian tumor groups by Kruskal–Wallis test and Mann–Whitney U-test as a post hoc, *P < 0.05, **P < 0.01, ***P < 0.001. Panel (A), all cases (n = 53). Panel (B), all mucinous tumors excluded (n = 29).

3.2. Ki67 and p53 related to tumor type and steroid receptor

As expected, mean Ki67 index as well as p53 immunoreactivity were elevated in ovarian tumors compared to normal ovaries (Fig. 4A–D). There was also a positive correlation between Ki67 and p53 staining intensity (r = 0.65, P < 0.001, SRC). When all patients were sub-divided according to steroid receptor (ERα, ERβ, PR), immunoreactivity score (0–3) in one group with high intensity (>1.5) and one group with low intensity (≤1.5), Ki67 index was not associated with ERα immunoreactivity. However, Ki67 was significantly greater in patients with low (1.5, n = 35) than high (>1.5, n = 18) ERβ immunoreactivity, 25.8 ± 3.6% versus 6.9 ± 3.1%, P < 0.001, MWU. For PR immunoreactivity similar differences were seen, 23.7 ± 3.4% versus 9.4 ± 4.6%, P < 0.01, n = 37 and 16, respectively. No statistically significant relations were found between p53 and indices of ERα, ERβ or PR immunoreactivity.

4. Discussion

This study confirms an abundant presence of not only the classic ER (ERα) and PR, but also the recently discovered ER isoform, ERβ, in normal ovaries and in ovarian tumors. Enmark et al., 1997; Brandenberger et al., 1998; Pujol et al., 1998; Hillier et al., 1998; Lau et al., 1999b; Fujimura et al., 2001; Hosokawa et al., 2001. A simultaneous expression of ERα, ERβ and PR in epithelial cells of all histopathological tumor types was noted, except for mucinous tumors (independent of histopathological classification, i.e., benign, borderline or malignant). In the present series of patients, it was clear that ERβ was expressed in all sampled ovarian specimens. However, the ERβ immunoreactivity was somewhat lower in mucinous tumors than in serous tumors. Stromal cells of mucinous tumors do contain ERα, ERβ and PR, but the ERβ expression pattern sharply contrasted to the ERα and PR-negativity found in epithelial cells in all mucinous tumors studied (benign, borderline or malignant). This finding was further corroborated in an additional series of 102 mucinous tumors in which epithelial cells were all ERα- and PR-negative (preliminary data). Absent ERα and PR immunoreactivity in mucinous tumors has been reported earlier (Fred et al., 1983; Abu-Jawdeh et al., 1996). However, it should be noted that a low degree of ERα and PR positivity in mucinous as compared to other epithelial ovarian tumors (Agarwal et al., 1987; Shiohara et al., 1997), as well as a high expression, have been reported (Akahira et al., 2000; Fujimura et al., 2001). The reasons underlying these divergent results are presently unclear, but may be due to differences in immunohistochemical procedures such as different epitope-specificity of antibodies used or histological misclassifications, e.g., mixed epithelial tumors or between different subtypes of mucinous tumors, e.g., endo-cervical/intestinal.
In breast cancer tumors it is generally accepted that ERα abundance is low in normal resting mammary glands and increases with proliferation in benign breast disease with atypia and ductal carcinoma in situ (Roger et al., 2000, 2001). In the present study, a significantly lower degree of ERβ immunoreactivity was found in epithelial cells of ovarian malignant tumors than in normal ovaries. A lower PR immunoreactivity was also found in epithelial cells of ovarian malignant tumors as compared with normal ovaries, whereas ERα levels remained unchanged between the different tumor groups, when excluding the mucinous tumors which were all ERα- and PR-negative. Attempts to quantify mRNA transcripts have demonstrated similar to slightly increased ERα levels (Brandenberger et al., 1998) in epithelial cells in ovarian malignancies as compared to normal ovarian tissues. Comparisons of the relation between ERβ and ERα in the present study showed a decreased ERβ/ERα ratio in ovarian epithelial cells of malignant tumors (after exclusion of ERα-negative mucinous tumors). Such calculated ratios have earlier been reported from studies where mRNA transcripts were measured. Results have indicated differences ranging from a uniform expression of both ERβ and ERα in normal ovarian surface epithelial cells (Lau et al., 1999b) and in unspecified normal ovarian tissue (Emmark et al., 1997; Brandenberger et al., 1998), to a predominant expression when measured with reverse transcription-polymerase chain reaction (Pujol et al., 1998).

A decreased ratio of ERβ/ERα mRNA as well as protein, supporting the finding herein, was found in a smaller series of patients with ovarian malignancies (Pujol et al., 1998; Brandenberger et al., 1998) and in primary cell cultures of human ovarian surface epithelium/ovarian cancer (Li et al., 2003). Nonetheless, comparisons between studies are shrouded by methodological differences, i.e., immuno-histochemistry with a cellular specificity versus mRNA measurement of mixed stromal and epithelial tissue. Additional studies with greater number of subjects using standardized methods for quantification of the different splicing variants of receptor mRNA transcripts and quantitative immunoblotting or receptor affinity methods are needed to further elucidate alterations in the numbers of functional gonadal steroid receptors in the different classes of ovarian tumors.

Ki67 nuclear antigen is related to cell proliferation (Gerdes et al., 1984). The normal p53 (wild type) is known as a tumor suppressor gene, and genetically deranged p53, by mutation or deletion of the p53 gene, is the most common genetic alteration described in human cancer cells and is present in high amount of ovarian tumors (Steele et al., 1998). As expected, Ki67 and p53 overexpression were positively correlated to each other and were markedly elevated in borderline and malignant ovarian tumors as compared with...
normal ovaries and benign ovarian tumors. Interestingly, Ki67 index was inversely correlated to PR and ER expression, whereas no such relation was found between p53 and expression of the tested steroid receptors. Estrogens have been shown to stimulate cell proliferation in cells containing ERs, and use of estrogen replacement therapy has been suggested to cause an increased risk for ovarian cancer in several studies (Clinton and Hua, 1997; Garg et al., 1998; Emmons and Kavanagh, 1999; Rodriguez et al., 2001). Paradoxically, estrogens have been proposed to exert a protective role against ovarian cancer (Risch, 1998; Lau et al., 1999b), possibly due to a decreased gonadotropin stimulation. Alternatively, oral contraceptives may diminish the risk of pre-malignant genetic abnormalities by decreasing the number of ovulations which, at subsequent stages, would otherwise respond to ER-mediated estrogen action. Contrary to estrogens, progesterone has been proposed to exert a protective role against ovarian cancer (Risch, 1998; Lau et al., 1999b). Epidemiological evidence has demonstrated a reduced risk of ovarian cancer for those women using progestin only contraceptives (Rosenberg et al., 1994). Furthermore, risk reduction for ovarian cancer seems to be greater with combined oral contraceptive pills with high-progestin potency than with low-progestin potency (Schildkraut et al., 2002). Decreased PR expression is found in ovarian malignancies (Noguchi et al., 1993; Lau et al., 1999b), and PR expression is related to improved survival in ovarian cancer patients (Miller and Langdon, 1997; Langdon et al., 1998; Munstedt et al., 2000; Lindgren et al., 2001), possibly through mechanisms of a progesterone-mediated inhibition of cell proliferation and induced apoptosis (Bu et al., 1997; Rodriguez et al., 1998).

In summary, ERβ and PR, but not ERα, epithelial immunoreactivity decreases with ovarian carcinogenesis. Several findings in the present study are in good agreement with the hypothesis that estrogen may stimulate ovarian tumor cell proliferation. The novel finding that all tested mucinous ovarian tumors retained ER expression, but not ERα or PR expression, may provide additional diagnostic and possibly therapeutic opportunities with anti-estrogens or SERMs targeting ERβ.

Acknowledgements
The authors wish to thank Ms. Inga-Lis Fransson for skillful technical assistance. This work was supported by grants from The Swedish Medical Research Council #13144 (JO), The Swedish Society of Medicine, The Research Foundation of the Department of Radiation Sciences/Oncology, Umeå University, Sweden.

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