

## Differential Expression of Progesterone Receptor Isoforms A and B in the Normal Ovary, and in Benign, Borderline, and Malignant Ovarian Tumors

Jun-ichi Akahira,<sup>1,2</sup> Takashi Suzuki,<sup>2</sup> Kiyoshi Ito,<sup>1</sup> Chika Kaneko,<sup>2</sup> Andrew D. Darnel,<sup>2</sup> Takuya Moriya,<sup>2</sup> Kunihiro Okamura,<sup>1</sup> Nobuo Yaegashi<sup>1</sup> and Hironobu Sasano<sup>2</sup>

<sup>1</sup>Departments of Obstetrics and Gynecology, and <sup>2</sup>Pathology, Tohoku University School of Medicine, 1-1 Seiryomachi, Aoba-ku, Sendai 980-8574

Human epithelial ovarian neoplasm is well-known to be sex steroid-related, but the possible biological significance of progesterone actions in these tumors remains controversial. In this study, we examined the differential expression patterns of the two progesterone receptor (PR) isoforms, PRA and PRB, using immunohistochemistry and real-time quantitative RT-PCR in normal and neoplastic ovarian tissues, and in cell lines derived from a normal ovarian surface epithelium and an ovarian epithelial carcinoma in order to further elucidate the possible involvement of progesterone in the development of ovarian neoplasms. The median *H* scores for PR isoforms in normal (*n*=8), benign (*n*=10), borderline (*n*=8) and malignant (*n*=24) ovarian tissues were as follows; PRA: 194.0, 171.0, 49.5, 0 (*P*<0.05), and PRB: 175.0, 180.5, 251.5, 168.5, respectively. In ovarian cancer cell lines (OVCAR-3 and Caov-3), the PRB/PRA mRNA ratio was increased by 17 $\beta$ -estradiol, both time- and dose-dependently. However, this ratio was unaltered following the addition of 17 $\beta$ -estradiol in a normal ovarian epithelial cell line (NOV-31). Immunoblotting analysis demonstrated that PRB protein expression was markedly up-regulated in OVCAR-3, whereas the PRA and PRB isoforms both appeared to be increased in NOV-31. These results suggest that down-regulation of PRA is associated with the development of ovarian epithelial carcinoma.

Key words: Ovary—Cancer—Immunohistochemistry—Quantitative RT-PCR—Progesterone receptor

Epithelial ovarian carcinoma is the leading cause of death from gynecological malignancies in the great majority of developed countries.<sup>1,2</sup> Both progesterone receptor (PR) and estrogen receptor (ER) have been reported in human epithelial ovarian carcinoma.<sup>3–5</sup> Various epidemiological data indicate that both endogenous and exogenous progesterone may play important roles in the pathogenesis and/or development of human ovarian neoplasms.<sup>6</sup> Previous studies have reported a reduction in ovarian cancer risk in postmenopausal women by using combination (estrogens plus progestins) hormone replacement therapy.<sup>7,8</sup> In addition, plasma levels of progesterone have been demonstrated to correlate well with ovarian cancer incidence.<sup>9,10</sup> These findings all suggest that PR is likely involved in the development and progression of this disease, but the mechanisms have yet to be fully characterized.

PR is a member of a subgroup of nuclear receptors which regulate a number of physiological and morphological processes in response to binding of their ligands.<sup>11</sup> There are two isoforms of the human PR, PRA and PRB, which differ only in that the smaller isoform, PRA, lacks the N-terminal 164 amino acids of the larger isoform, PRB.<sup>12–15</sup> PRA and PRB are both products of a single gene and are translated from individual messenger RNA species under the control of distinct promoters.<sup>15</sup> The transcription of both isoforms is indirectly induced by estro-

diol via ER.<sup>15</sup> Both PRA and PRB function as ligand-activated transcription factors, but several *in vitro* studies have demonstrated different functional characteristics between these two isoforms. Transcriptional activation of the progesterone-responsive element-containing promoters by PRB is more marked than that by PRA, although the differences are cell-specific.<sup>15–17</sup> In addition, PRA can also act as a transcriptional inhibitor of other steroid hormone receptors, including ER and PRB.<sup>16–19</sup> The results of these studies all indicate that the relative levels of PRA and PRB within target cells may determine the functional responses to progesterone.

Epithelial ovarian cancers are considered to arise from the ovarian surface epithelium, which shares a common embryonic origin with epithelia of Mullerian duct-derived tissues (fallopian tube, endometrium, and endocervix), but is different from the granulosa-thecal cells of the ovary.<sup>20,21</sup> Borderline tumors or carcinomas of low malignant potential of the ovary are considered to contain features of both benign and frank invasive malignant tumors, because they exhibit nuclear atypia and marked proliferation, but not the invasive properties seen in frank invasive carcinoma.<sup>22</sup> However, little is known regarding the expression patterns of the two PR isoforms in these various histological types of ovarian neoplasms. Therefore, we first evaluated the expression of PRA and PRB using immunohistochemistry in these tumors.

Estrogen is known to be a potent regulator of PR expression, though its mode of PR regulation has been

demonstrated to be different between normal and carcinomatous tissues in the human breast.<sup>25)</sup> To date, however, PR regulation has not been examined in detail in the human ovary and its neoplasms. Therefore, we evaluated the expression and regulation of these PR isoforms by estradiol in cell lines derived from ovarian carcinoma and surface epithelium using real-time quantitative PCR and immunoblotting.

## MATERIALS AND METHODS

**Tissues and cell lines** The ovarian cancer cell lines, OVCAR-3<sup>24)</sup> and Caov-3 were cultured in RPMI-1640 medium (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. A normal ovarian surface epithelial cell line, NOV-31,<sup>25)</sup> was maintained in a mixture of MCDB105 medium and Medium 199 (Sigma, Saint Louis, MO) supplemented with 15% fetal bovine serum. In experiments in which the effects of estrogen were measured, cells were first cultured in medium depleted of steroids (withdrawal medium) which consisted of phenol red-free minimal essential medium (Gibco-BRL) supplemented with charcoal-treated fetal bovine serum.

Eight normal ovaries, 10 benign ovarian cysts (serous cystadenoma), 8 borderline tumors (serous adenocarcinoma with low malignant potential) and 24 ovarian cancer cases (serous adenocarcinoma) were obtained from patients following surgical therapy from 1990 to 1998. All of these archival specimens were retrieved from the surgical pathology files at our institution (Department of Obstetrics and Gynecology, Tohoku University School of Medicine, Sendai). These specimens were all fixed in 10% formalin and embedded in paraffin. One part of the ovarian cancer tissue was snap-frozen in liquid nitrogen and then stored at  $-80^{\circ}\text{C}$  in our laboratory until further use. The research protocol was approved by the ethics committee of Tohoku University School of Medicine.

**Immunohistochemistry** Monoclonal antibodies for PR (hPRA2 and hPRA7 for immunohistochemistry, and hPRA3 for immunoblotting) were purchased from NeoMarkers (Fremont, CA). The hPRA7 and hPRA2 antibodies recognize PRA and PRB, respectively. The hPRA7 antibody employed in this study recognizes both PRA and PRB on immunoblot analysis,<sup>26)</sup> but specifically recognizes PRA when utilized for immunohistochemistry.<sup>27)</sup> Both antibodies for immunohistochemistry were diluted to 1:100 in our study. Immunohistochemical analysis was performed with the streptavidin-biotin amplification method using a Histofine Kit (Nichirei, Tokyo), as previously described in detail.<sup>28)</sup> For immunostaining of PRA and PRB, the slides were heated in an autoclave at  $120^{\circ}\text{C}$  for 5 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate, pH 6.0) following deparaffinization. The anti-

gen-antibody complex was visualized with 3,3'-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris-HCl buffer (pH 7.6), and 0.006%  $\text{H}_2\text{O}_2$ ), and counterstained with hematoxylin. Tissue sections of normal proliferative endometrium were used as positive controls for PRA and PRB.<sup>26)</sup> As a negative control, normal rabbit or mouse IgG was used instead of the primary antibodies, and no specific immunoreactivity was detected in these tissue sections.

**Scoring of immunohistochemistry** Both relative immunointensity and distribution of PR isoforms were assessed in a semiquantitative manner by two of the authors (J. A. and T. M.) using the *H* score system reported by McCarty *et al.*<sup>29)</sup> Scores were generated as follows: ( $3 \times$ [percentage of strongly staining cells]) + ( $2 \times$ [percentage of moderately staining cells]) + ( $1 \times$ [percentage of weakly staining cells]). This scoring system yielded results ranging from 0 to 300. Evaluation was carried out independently for at least 500 cells, and discordant cases (interobserver difference of  $>5\%$ ) were re-evaluated simultaneously by the two observers using a double-headed light microscope.

**RNA preparation and reverse transcription** Cells were grown to 70% confluence in 10-cm plates, then incubated with withdrawal medium for 48 h, and treated for 2 h to 24 h with  $10^{-8}$  M  $17\beta$ -estradiol (Sigma) or with  $17\beta$ -estradiol at concentrations ranging from  $10^{-11}$  to  $10^{-7}$  M for 24 h. Total RNA was isolated from tissues and cell lines by phenol-chloroform extraction using Isogen (Nippon Gene). A RT-PCR kit (Superscript Preamplification system, Gibco-BRL) was employed and cDNA synthesis was carried out according to the instructions. cDNAs were synthesized from 1  $\mu\text{g}$  of total RNA using random hexamer and Superscript II reverse transcriptase at  $42^{\circ}\text{C}$  for 50 min. One microliter of this reaction was analyzed by real-time PCR.

**PCR primers and control plasmids** PRA mRNA cannot be distinguished from PRB by RT-PCR because PRA has no specific sequence to distinguish it from PRB mRNA. Thus we used a primer set termed 'PRAB' to detect products that contain both PRA and PRB cDNA. Primers for subsequent PCR were as follows: PRAB, 5' sense-CCTCGACACCTTGCTGAA and 3' antisense-CGCCAACA-GAGTGTCCAAGAC (1576–1794, 239 bp); PRB, 5' sense-TAGTGAGGGGGCAGTGGAAAC and 3' antisense-AGGAGGGGGTTTCGGGAATA (955–1377, 442 bp);  $\beta$ -actin, 5' sense-CCAACCGCGAGAAGATGAC and 3' antisense-GGAAGGAAGGCTGGAAGAGT (382–841, 459 bp). Control plasmids, as a standard to quantify the PR isoforms, were generated as follows; the PCR products from normal endometrium were electrophoresed and the bands consistent with the predicted size of PR isoforms were purified with a GFX PCR DNA/Gel Band Purification Kit (Amersham, Arlington Heights, IL). They were then subcloned into a plasmid using T4 DNA ligase (TaKaRa, Tokyo). Following subcloning, the plasmid

together with the PR PCR insert was purified with a Qiagen plasmid Maxi kit (Qiagen, Valencia, CA) and subsequently sequenced by the dideoxynucleotide chain termination method according to the manufacturer's recommendations (ABI Prism dRhodamine terminator cycle sequencing ready mix, Applied Biosystems, Inc., Foster City, CA). The DNA concentration was assessed by spectrophotometry at 260 nm and determined as an average of two measurements.

**Real-time quantitative RT-PCR** Real-time quantitative PCR was performed using the "Light Cycler System" (Roche Diagnostics, Mannheim, Germany). For the determination of PRAB and PRB cDNA content, a 20.16  $\mu$ l reaction mixture consisting of 2  $\mu$ l of 10 $\times$  LC-DNA Master Mix SYBR Green I (Roche Diagnostics), 0.16  $\mu$ l of Anti-Taq reagent, 2  $\mu$ l of 10  $\mu$ M primers, 2.4  $\mu$ l of 25 mM MgCl<sub>2</sub> (for PRB, 1.6  $\mu$ l), 12.6  $\mu$ l of H<sub>2</sub>O (for PRB, 13.4  $\mu$ l), and 1  $\mu$ l of template cDNA was prepared. An initial denaturation step at 95°C for 10 s was followed by 40 cycles of denaturation at 95°C for 0 s, annealing at 60°C (for PRB, 59°C) for 10 s, and elongation at 72°C for 10 s. The fluorescence intensity of the double-strand specific SYBR Green I, reflecting the amount of formed PCR-product, was obtained at 87°C after the end of each elongation step.  $\beta$ -Actin cDNA fragments were amplified as positive controls. Negative controls without RNA and without reverse transcriptase were also performed. Two independent RT-PCR reactions were performed for each sample.

**Whole cell protein extraction** Cells were grown to 70% confluence in 10-cm plates, and then incubated with withdrawal medium for 48 h, and treated with either vehicle (ethanol) or 10<sup>-8</sup> M 17 $\beta$ -estradiol dissolved in ethanol for 72 h. Following removal of the culture medium with phosphate-buffered saline (PBS), whole cell protein was extracted using "M-PER" Reagent (Pierce, Rockford, IL). The protein concentration of the supernatant was measured utilizing a BCA protein assay kit (Pierce).

**Immunoblotting** An ECL-Plus Western blotting analysis system was purchased from Amersham, and the supplied kit instructions were followed. Protein samples (5  $\mu$ g) were mixed with an equal volume of 2 $\times$  concentrated SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, boiled, and then electrophoresed on 7% polyacrylamide gels containing SDS. Proteins were then transferred to a nitrocellulose membrane (Hybond PDVF). The membranes were incubated in blocking solution (PBS containing 5% nonfat milk and 0.05% Tween-20), and then incubated in a 1:100 dilution of hPRA-3 antibody in blocking solution overnight at 4°C. The membranes were washed thoroughly in water and then incubated for 60 min at room temperature in a 1:50 000 dilution of sheep anti-mouse horseradish peroxidase-conjugated secondary antibody followed by ECL-plus detection reagents (Amersham). Actin

was also detected for each sample as a positive control. At least two independent experiments were performed for each sample.

## RESULTS

**Immunohistochemistry** Immunoreactivity for PR isoforms was confined exclusively to the nuclei of epithelial cells (Fig. 1). The median *H* scores for PR isoforms in normal, benign, borderline and malignant ovarian tissues were as follows; PRA, 194.0 (range 160–210), 171.0 (138–183), 49.5 (18–72), 0 (0–84) ( $P < 0.05$  by Kruskal-Wallis test), and PRB, 175.0 (147–210), 180.5 (120–275), 251.5 (201–270), 168.5 (50–265), respectively (Fig. 2). In the normal ovarian surface epithelium and adenoma, PRA and PRB appeared to be equally expressed, whereas in borderline and malignant tissues, PRB was the main PR isoform (Figs. 1, 2). The percentage of positive cases, which were defined as those with an *H* score of more than 30, in normal, benign, borderline and malignant ovarian tissues were as follows; PRA, 100%, 100%, 50%, 25% ( $P < 0.05$ ), and PRB, 100%, 100%, 100%, 95.8%, respectively.

**Quantitative RT-PCR** The baseline expression of PR mRNAs in NOV-31, OVCAR-3, Caov-3 and ovarian cancer tissues are summarized in Fig. 3. PRB mRNA was detected in all cell lines examined and ovarian cancer tissues. Expression of both ER $\alpha$  and  $\beta$  mRNA was detected in all cell lines (data not shown).

Amplification of increasing amounts of plasmid (diluted in water from 0.02 to 200 pg) containing the target sequence was performed (Fig. 4a). Standard curves of the fluorescence intensity plotted against the logarithm of the plasmid concentration were constructed for each plasmid (Fig. 4b). To confirm the accuracy of experiments, the error rate of standard curves were controlled within 0.2 in each experiment according to the manufacturer's recommendation (Fig. 4b). mRNA integrity was controlled by amplification of the  *$\beta$ -actin housekeeping* gene. In OVCAR-3 and Caov-3, the PRB/PRAB mRNA ratio was strongly stimulated by 17 $\beta$ -estradiol in a time- and dose-dependent manner, but this ratio was not altered in the NOV-31 cell line (Fig. 5).

**Immunoblotting** Two immunoreactive bands corresponding to PRA and PRB were detected in the blot (Fig. 6). Following treatment with 17 $\beta$ -estradiol for 3 days, PRB protein expression was markedly up-regulated in OVCAR-3, whereas both PRA and PRB appeared to be increased in the NOV-31 cell line (Fig. 6).

## DISCUSSION

Results from our present study demonstrated that the expression of PRA decreased from non-neoplastic to

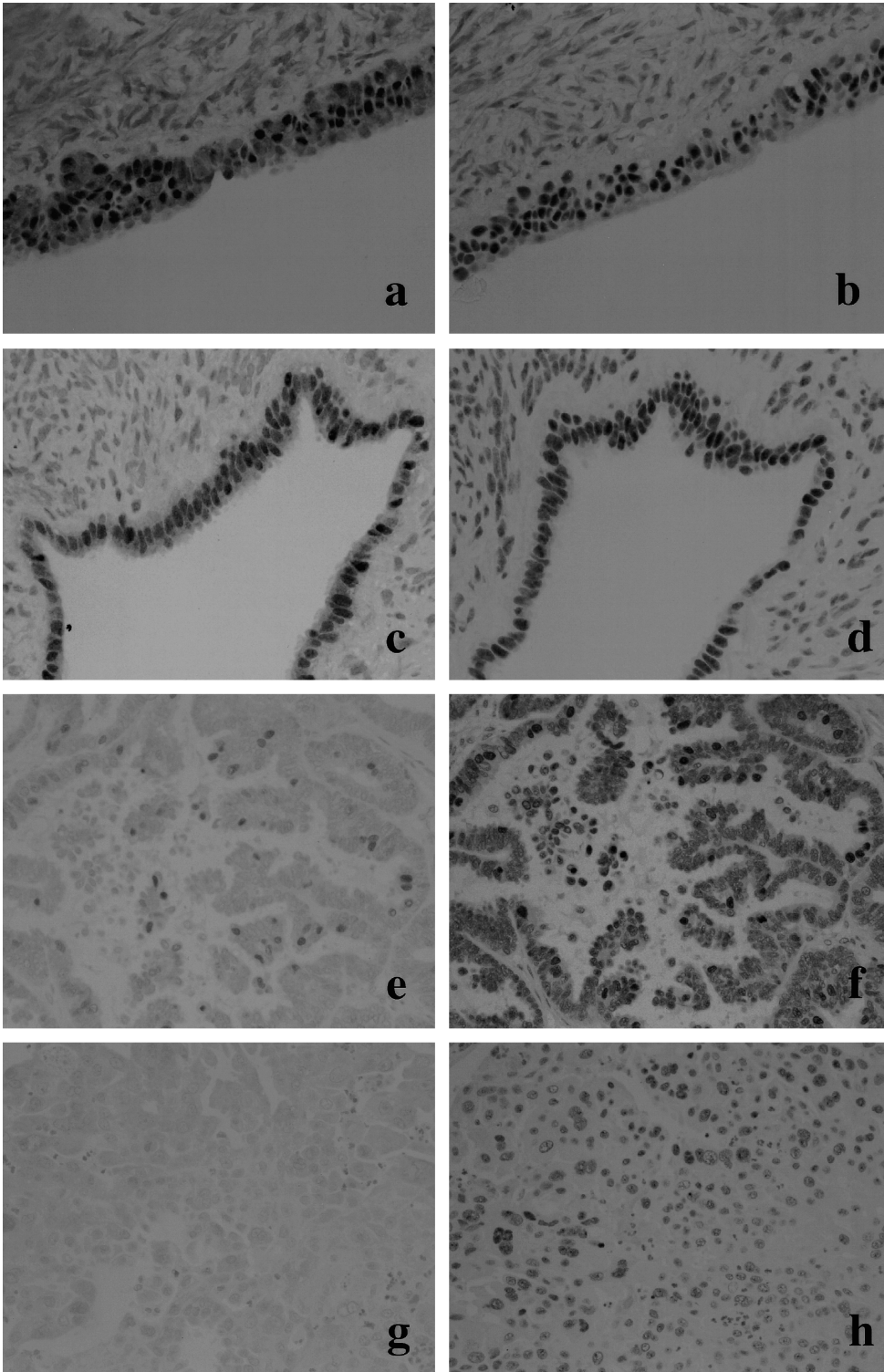


Fig. 1. Immunohistochemistry of serial tissue sections for PRA (a, c, e, g) and PRB (b, d, f, h) in the ovarian surface epithelium (a, b), serous adenoma (c, d), serous adenoma of borderline malignancy (e, f), and serous adenocarcinoma (g, h). Note that the expression of PRA appears to become weaker with malignant transformation, although the expression of PRB seems constant. (Original magnification  $\times 200$ )

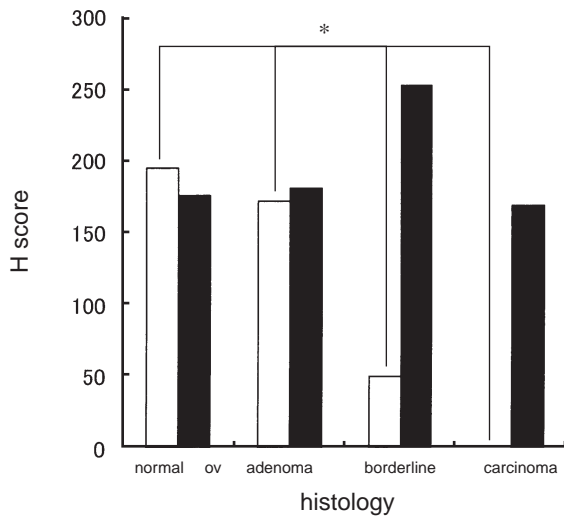


Fig. 2. Histogram of PR isoform expression from normal to malignant ovarian tissues. *H* score was obtained as described in "Materials and Methods." \*  $P < 0.05$  by Kruskal-Wallis test. □ PRA, ■ PRB.

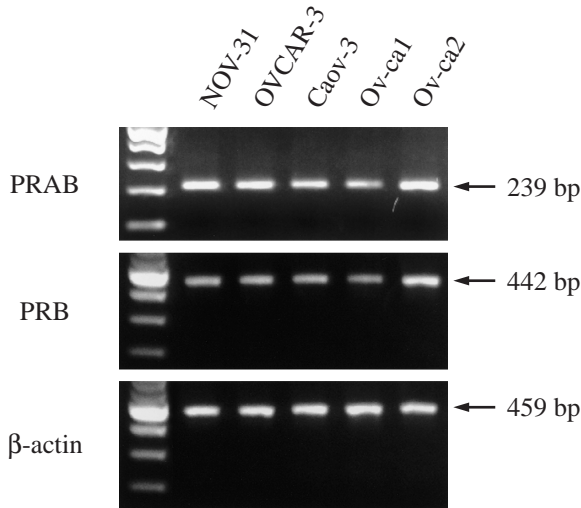


Fig. 3. Baseline expression of PR mRNA in cell lines and tissues. RT-PCR analysis of total RNA extracted from NOV-31, OVCAR-3, Caov-3 and two serous adenocarcinoma tissues (Ovca 1 and Ovca 2). Specific bands consistent with PRAB (239 bp) and PRB (442 bp) were detected in all samples examined.  $\beta$ -Actin is shown as a positive control.

malignant ovarian tissues. This decrease was especially marked between benign and borderline ovarian tumors, whereas PRB maintained a constant level of expression in both neoplastic and non-neoplastic ovarian tissues. In

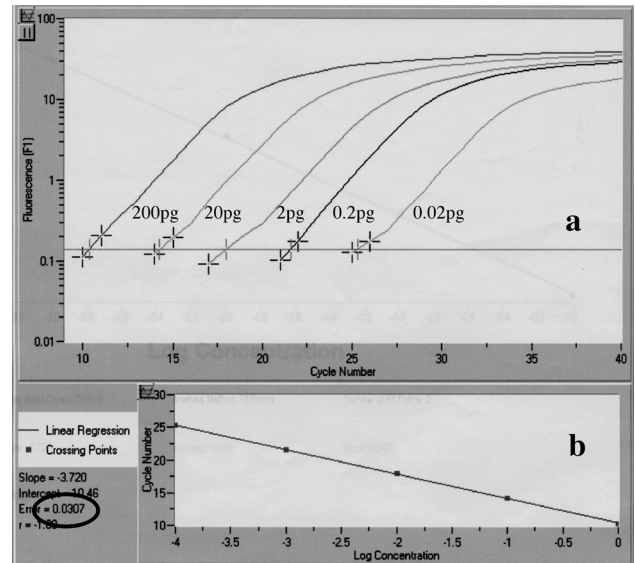


Fig. 4. Amplification (a) and standard curve (b) obtained with the control plasmid of PRAB. Serial 10-fold dilutions from 200 to 0.02 pg of plasmids that contain target sequences were made in water. Fluorescence intensity was plotted against the logarithm of the plasmid concentration to construct a standard curve. The error rate (circle) in all standard curves was controlled within 0.2 in each experiment.

addition, PRB mRNA expression levels relative to that of PRA were stimulated time- and dose-dependently by addition of estrogen in ovarian cancer cell lines. These findings all suggest that PRA is down-regulated transcriptionally in ovarian carcinoma compared to the normal ovary, and suggest the importance of differential patterns of PR isoform expression in ovarian carcinogenesis.

Several previous studies indicated a possible correlation between PR expression and ovarian carcinogenesis. Zeimet *et al.* reported that virtually all specimens of normal ovarian epithelium contained PR.<sup>30)</sup> In another study, 93% of malignant ovarian tumors exhibited PR immunonegativity, whereas PR immunopositivity was detected in the majority of borderline tumors.<sup>31)</sup> In ovarian cancer, several authors reported that the higher PR level was correlated with a favorable clinical outcome and well-differentiated state.<sup>32-34)</sup> Recently, Lau *et al.* reported PR mRNA expression in normal surface epithelial cells and a marked down-regulation of PR transcript expression in most ovarian cancer cells.<sup>35)</sup> Lau *et al.* also reported that the loss of PR mRNA expression in surface epithelial cells may be responsible for neoplastic transformation. The results of these studies are consistent with those in our study, i.e., overall LI of PR (PRA+PRB) decreased throughout the process of malignant transformation. However, in all previous studies of PR in ovarian tissues, PR isoforms were

not evaluated separately as in our present study. The analysis of subtypes of PR has provided new insights into the possible biological roles of PR in many normal and pathological tissues. Ovarian surface epithelial cells derived from postmenopausal women expressed substantial levels of both PR isoforms. These findings suggest that sex steroid responsiveness is retained in surface epithelial cells

following menopause and may explain the protective effects of progesterone against ovarian cancer.<sup>6, 7, 10</sup> Loss of progesterone regulation in surface epithelial cells may also be involved in the development of ovarian cancers that increase in incidence in women after the age of 45. Our results, together with the findings of previous studies, suggest that a decreased expression of PR, especially that of PRA, plays an important role in the development of ovarian neoplasms.

It is well known that PR is regulated by estrogen via ER.<sup>15</sup> The biological effects of estrogens are, however, not restricted to the well known induction of PR levels, but most importantly, include alteration of the ratio of the two PR isoforms A and B, which are known to possess different biological functions.<sup>36, 37</sup> Preferential up-regulation of PRB by estrogen has been reported in the T47D human breast carcinoma cell line,<sup>37</sup> and in human endometrial tissues.<sup>38</sup> However, the stimulatory effects of estrogen on PRA protein levels have been reported to be greater than those on PRB in the chicken oviduct,<sup>36</sup> suggesting that estrogen stimulation of PRA and PRB is likely to be cell, tissue and species-specific. Our results suggest that an increase of the PRB/PRAB mRNA ratio by estrogen may be a feature of ovarian carcinoma cells, and further indicate that ovarian carcinoma cells are associated with down-regulation of PRA, although this requires confirmation. Further investigations are required to clarify the mechanisms of regulation of PRA and PRB in human ovarian epithelial carcinomas.

In contrast to breast or endometrial carcinoma, in which steroid hormone receptor status is well correlated with

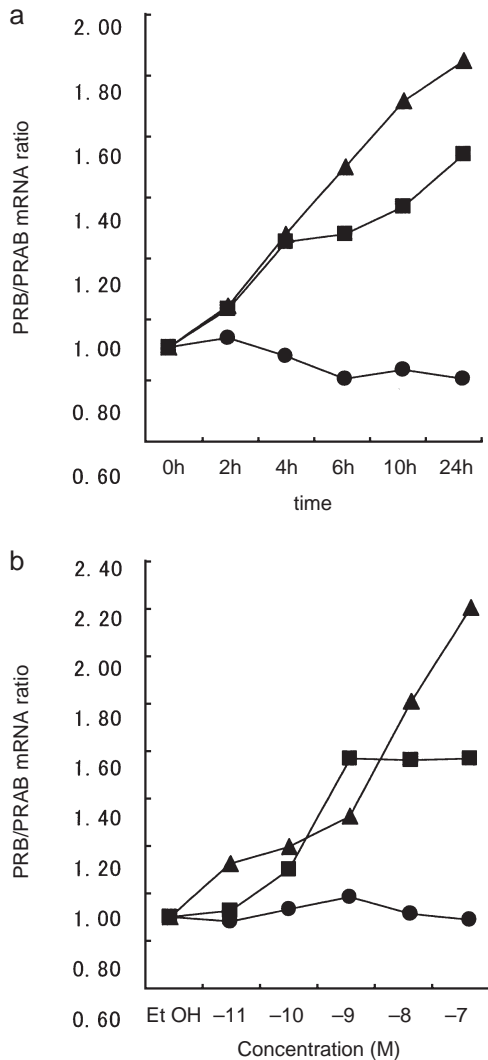


Fig. 5. Results of PR regulation by 17 $\beta$ -estradiol in ovarian cancer cell lines, OVCAR-3, Caov-3 and normal surface epithelial cell line, NOV-31. Cells were treated with withdrawal medium for 48 h and then treated for various periods (a) with various concentrations (b) of 17 $\beta$ -estradiol. Total RNA was extracted, and the relative levels of PRB versus PRAB mRNA were detected by real-time quantitative RT-PCR. The expression levels of 0 h (a) and EtOH (b) were defined as 1. Each point represents the mean of two experiments. ● NOV-31, ■ Caov-3, ▲ OVCAR-3.

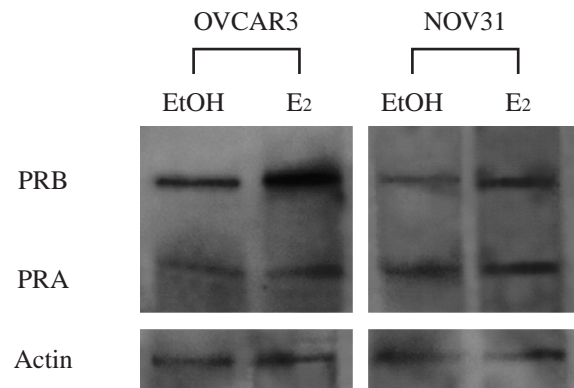


Fig. 6. Results of immunoblot analysis for PR isoforms in NOV-31 and OVCAR-3 ovarian cell lines. Cells were treated with withdrawal medium for 48 h and then treated with 17 $\beta$ -estradiol for 72 h. Whole cell proteins were extracted, and immunoblotting was performed as described in "Materials and Methods." Note the strong up-regulation of PRB by estrogen in OVCAR-3. Actin is shown as a positive control.

response to hormonal manipulation,<sup>39)</sup> the impact of tumor receptor status and response to hormonal therapy among patients with epithelial ovarian cancer has not been well-examined. Several clinical trials with progestins have demonstrated impressive response rates of up to 50%, but most previous clinical trials reported response rates of around 10–20%.<sup>39,40)</sup> In breast carcinoma, Graham *et al.* found that PRA exceeded PRB in 76% of the cases among the PR isoforms.<sup>41)</sup> They also reported that tumors containing primarily PRA may correspond to a subset of patients with low or aberrant response to endocrine agents.<sup>42)</sup> Ariga *et al.* reported that PRA is dominantly expressed in breast carcinoma, and is associated with histological grade.<sup>43)</sup> Kumar *et al.* examined the expression of PRA and PRB in endometrial carcinoma and reported that selective down-regulation of PRB may represent an insufficient response to progestin therapy in patients with poorly differentiated endometrial carcinoma.<sup>44)</sup> Recently, Sasaki *et al.* reported selective methylation of PRB and its role in the dominant expression of PRA detected in endometrial cancer.<sup>45)</sup> In contrast, we showed previously that in ovarian cancer, PRB is dominantly expressed.<sup>32)</sup> The discrepancies of PR isoform expression between breast/endometrial and ovarian cancer may explain the different responses to progestin therapy reported in the various clinical trials as noted above. In PRA-knockout mice, the antiproliferative effect of progesterone for endometrium is absent, as in PR-knockout mice.<sup>46)</sup> Mulac-Jericevic *et al.* also reported that

selective ablation of PRA may result in a gain of progesterone-dependent proliferative activity mediated via PRB. These results suggest that introduction of a PRA-selective progestin that can distinguish between different conformations of PRA and PRB may be of significant clinical value for therapy or prophylaxis of various sex steroid-dependent neoplasms. There are no 'selective progesterone receptor modulators' as with estrogen, but the above approach may lead to a better understanding of the roles of steroid hormones and a basis for the selection of more effective endocrine treatment in these neoplasms.

In conclusion, the results of our present study suggest that down-regulation of PRA, relative to PRB, may be a potential marker of ovarian carcinogenesis. Relative differential expression of PRA and PRB isoforms is likely to be of critical importance to ensure appropriate reproductive tissue responses to progesterone. The emergence of progesterone resistance, via down-regulation or mutational inactivation of receptors, may be a key feature in ovarian epithelial transformation.

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