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Progesterone Receptor A and B Isoforms in the Human Breast and Its Disorders

Naohiro Ariga,[1,2,4] Takashi Suzuki,[1] Takuya Moriya,[1] Michio Kimura,[3] Tsukasa Inoue,[1] Noriaki Ohuchi[2] and Hironobu Sasano[1]

[1]Department of Pathology and [2]Department of Surgical Oncology, Tohoku University School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-8575 and [3]Department of Surgery, Tohoku Kosai Hospital, 2-1-1 Kokubun-cho, Aoba-ku, Sendai 980-0803

Two different isoforms of progesterone receptor (PR), PRA and PRB, are expressed in target tissues at comparable levels. In this study, we first examined PRA and PRB immunoreactivity in human breast cancer and various intraductal proliferative epithelial lesions, and correlated these findings with clinicopathologic parameters. We then examined mRNA expression of PRA and PRB in six cases of invasive ductal carcinoma using RT-PCR. Immunoreactivity for both PRA and PRB was positive in the great majority of proliferative disease without atypia (PDWA) (85% for PRA and 96% for PRB) and atypical ductal hyperplasia (ADH) (100% for PRA and 100% for PRB), but the ratio of immunopositive cases and immunohistochemical (IHC) scores was significantly smaller in ductal carcinoma *in situ* (DCIS) (65% for PRA and 75% for PRB) and invasive ductal carcinoma (IDC) (66% for PRA and 55% for PRB) than in PDWA and ADH. There was a significant positive correlation between IHC scores for PRA and estrogen receptor α (ERα) in IDC, DCIS and ADH but not between PRB and ERα. In IDC, both PRA and PRB IHC scores were significantly associated with histological grade, but there was no association between PRA or PRB status and lymph node involvement, tumor size, or prognosis of the patients. The expression of mRNAs for both PRA and PRB was detected in all six cases of IDC examined. These results suggest that both PRA and PRB are strongly associated with ERα in human breast and this relation may be disturbed in breast cancer.

Key words: Progesterone receptor A — Progesterone receptor B — Breast — Carcinoma — Immunohistochemistry

Human progesterone receptor (PR) exists as two isoforms, A and B. These isoforms are encoded by separate mRNAs which are transcribed from two distinct promoters, both of which are under estrogen control.[1,2] PRA and PRB are both expressed in progesterone target tissues at comparable levels. The ratio of PRA:PRB has been suggested to influence the biological actions of progesterone. Therefore, investigating the relative ratio of PR isoforms in progesterone-responsive tissues may provide important insights into the physiology and perhaps pathogenesis relating to progesterone-mediated actions.

In human breast cancer cells, PRA over-expression has been reported to be associated with an alteration in adhesive properties.[3] Previous studies using immunoblot analysis have demonstrated very high levels of PRA (up to 100 fold higher than PRB) in a subset of human breast tumors.[4] However, immunolocalization of PR isoform proteins has not been reported in detail in human breast cancer. Therefore, in this study, we first immunolocalized PRA and PRB in human breast cancer and intraductal epithelial proliferative lesions. We then examined the mRNAs for PRA and PRB in invasive ductal carcinoma cases using reverse transcription-polymerase chain reaction (RT-PCR) analysis. We also examined the correlation between these findings and clinicopathological factors of invasive ductal carcinoma including estrogen receptor (ER) α status, Ki67 labeling index (LI), histological grades, and lymph node status, in order to further characterize the biological significance of these PR isoforms in breast carcinoma.

[4] To whom correspondence should be addressed at the Department of Surgical Oncology, Tohoku University Hospital.
E-mail: n-ariga@patholo2.med.tohoku.ac.jp

MATERIALS AND METHODS

Cases Surgical pathology specimens were retrieved from the pathology files of Tohoku University Hospital, Sendai, Kawasaki University Hospital, Kurashiki, and Tohoku Kosai Hospital, Sendai. These specimens included 47 cases of invasive ductal carcinoma (IDC), 40 cases of ductal carcinoma *in situ* (DCIS), 27 cases of atypical ductal hyperplasia (ADH), and 27 cases of proliferative disease without atypia (PDWA) including moderate and florid hyperplasia of the usual type. Pathological diagnosis was based on the work of Dupont *et al.*[5] and of Ottesen *et al.*[6] Classification of DCIS was based on the Consensus Conference on the Classification of Ductal Carcinoma *In Situ* in 1997.[7] Non-pathological breast tissues were available for examination in 13, 12 and 12 cases of DCIS, ADH and PDWA, respectively. All of these specimens were fixed in

10% formalin for 24 to 48 h and were embedded in paraffin. Portions of carcinoma specimens were frozen in liquid nitrogen and stored at −80°C until use for RT-PCR analysis. The research protocol for this study was approved by the ethics committee of Tohoku University School of Medicine, Sendai. Clinical data, including age at surgery, tumor size and lymph node status for IDC cases were retrieved from patients' charts.

Histological grading of cancer For the grading of IDC, the Nottingham classification[8] was used. Grades of differentiation include grades I, II, and III. In this study, grade I and grade II are designated low grade (non-high grade), and grade III is considered to be high grade.[9, 10]

For grading of DCIS, the Van Nuys DCIS classification[11, 12] was used. In this study, group 3 was designated as high grade, and the other two groups were designated as low grade (non-high grade).[11]

Antibodies Monoclonal antibodies for PRA (hPRa7) and PRB (hPRa2) were purchased from NeoMarkers, Inc. (Union City, CA). hPRa7 can recognize both high (B) and low (A) MW forms of human PR, but this antibody has been reported to recognize only PRA in 10% formalin fixed and paraffin-embedded tissue sections.[13, 14] hPRa2 exclusively recognizes PRB.[13, 15] Antibodies against ERα and Ki67 antibody (MIB1) were commercially obtained. The source, optimal dilution, and pretreatment methods of immunostaining are summarized in Table I.

Immunohistochemistry Serial 3 μm thick sections were prepared. The first and last sections were stained with hematoxylin-eosin for confirmation of the pathological diagnosis. Sections from paraffin formaldehyde-fixed blocks were deparaffinized in xylene and dehydrated in a gradient of ethanol. After washing of these sections in distilled water, an antigen retrieving method was applied. Sections were subsequently washed in 0.01 *M* phosphate-buffered saline (PBS). Intrinsic peroxidase activity was blocked with 0.9% hydrogen peroxide in 0.01 *M* PBS for 10 min at room temperature. Sections were then incubated with 1% normal rabbit serum in PBS for 30 min at room temperature, followed by an overnight incubation with the primary antibody at 4°C. The dilutions of primary antibodies employed in this study are summarized in Table I. The sections were then incubated with biotinylated rabbit anti-mouse IgG (Histofine Kit; Nichirei, Tokyo), and with horseradish peroxidase-conjugated streptoavidin (Nichirei). Sections were developed with 3,3′-diaminobenzidine (DAB) and counterstained with hematoxylin. As a negative control for immunostaining, sections were incubated with 0.01 *M* PBS or normal mouse IgG, instead of primary antibodies. No specific immunoreactivity was detected in these tissue sections.

Scoring of immunoreactivity For evaluation of Ki67, immunostained slides were evaluated independently by two of the authors (N. A. and T. M.) in high-power fields (×400) using standard light microscopy. In each case, 200−500 cells in the lesion were counted, and the percentage of immuno-positive cells, i.e. LI, was determined. Immunoreactivities for ER and PR were assessed utilizing the same method, as described above. Because of variations of relative nuclear immunointensity of these receptor proteins among the cases examined, we utilized a quantitative method, based on the system reported by Allred *et al.*[16] In brief, an entire slide was evaluated by light microscopy. First, a "proportion score" was assigned, which represented the estimated proportion of positive-staining tumor cells (0, none; 1, <1/100; 2, 1/100 to 1/10; 3, 1/10 to 1/3; 4, 1/3 to 2/3; and 5, >2/3). An "intensity score" was then assigned, which represented the average intensity of positive tumor cells (0, none; 1, weak; 2, intermediate; and 3, strong). The proportion and intensity scores were subsequently added to obtain a total score, which ranged from 0 to 8. This total score was finally designated as an immunohistochemistry (IHC) score. Cases with disconcordant results between observers were simultaneously re-evaluated by the same two authors mentioned above using double-headed light microscopy. Based on the report by Harvey *et al.*,[17] an IHC score greater than three was considered positive.

RT-PCR RT-PCR was performed to confirm the expression of each isoform of PR using six IDC cases. Total RNA was extracted by homogenizing tissue specimens in guanidinium thiocyanate followed by ultracentrifugation in cesium chloride, as described previouly.[18] RNA was quantified spectrophotometrically at 260 nm. A RT-PCR kit (SUPERSCRIPT Preamplification system, Gibco-BRL, Grand Island, NY) was employed in the synthesis and

Table I. Summary of Primary Antibodies Employed in This Study

Antibodies	Dilution	Antigen retrieval	Source
PRA (clone hPRa7)	1:100	Autoclave[a]	NeoMarkers (Union City, CA)
PRB (clone hPRa2)	1:100	Autoclave[a]	NeoMarkers (Union City, CA)
ERα (clone ER1D5)	1:1 (prediluted)	Autoclave[a]	Immunotech (Marseille, France)
Ki67 (clone MIB1)	1:50	Microwave[b]	Immunotech (Marseille, France)

a) Autoclaved for 5 min at 120°C in 0.01 mol/liter sodium citrate buffer (pH 6.0).
b) Treated for 7.5 min in 0.01 mol/liter sodium citrate buffer (pH 6.0).

amplification of cDNA. cDNAs were synthesized from 5 μg of total RNA in 20 μl of reverse transcription buffer containing 50 m*M* Tris-HCl (pH 8.3), 55 m*M* KCl, 3 m*M* $MgCl_2$, 0.02 *M* DTT, 0.5 m*M* dNTP, and 62.5 mg/ml oligo(dT). Reverse transcription was carried out for 50 min at 42°C with SUPERSCRIPT II reverse transcriptase. The reaction mixture was subsequently inactivated for 15 min at 70°C. An aliquot of each reverse transcription reaction product (2 μl) was amplified with either PRA and B (PRAB), or PRB primers in a solution containing 1× PCR buffer, 1.5 m*M* $MgCl_2$, 0.1 m*M* dNTP and 1.25 U *Taq* DNA polymerase (PCR Reagent System, Gibco-BRL), in a total volume of 25 μl. This volume was overlaid with mineral oil and then incubated in a DNA thermal cycler (PTC-200 DNA Engine, MJ Research, Inc., Waltham, MA). A 35-cycle amplification profile consisted of denaturation at 94°C for 45 s, annealing at 55°C for 30 s, and extension at 72°C for 1.5 min. The resulting products were then subjected to gel electrophoresis and visualized by ethidium bromide staining. Primers for PCR reactions were as follows; PRB[19]: 5′ sense-ACAGAATTCATGACTGAGCTGAAGGCAAAGGGT and 3′ antisense-ACAAGATCTCAAACAGGCACCAAGAGCTGCTGA (744–1173, 429 bp); PRAB[19]: 5′ sense-ACAGAATTCATGAGCCGGTCCGGGTGCAAG and 3′ antisense-ACAAGATCTCCACCCAGAGCCCGAGGTTT (1239–1482, 243 bp); β-actin[20]: 5′ sense-GATTCCTATGTGGGCGACGAG and 3′ antisense-CCATCTCTTGCTCGAGTC (192–723, 532 bp). Human β-actin primers were utilized as positive controls. Negative controls without RNA and without reverse transcriptase were also performed.

Statistical analyses A Kruskal-Wallis test was used for comparison of three or more groups, for continuous variables. Scheffe's test was used as a multiple comparison post test. Mann-Whitney's *U* test was used in the comparison of two groups with continuous variables. χ^2 test or Fisher's exact test was used in the comparison of calculated data for some categories. The correlation between different parameters with continuous variables was assessed in terms of Spearman's rank-order correlation coefficient. $P<0.05$ was considered significant. All *P* values were from two-sided tests.

RESULTS

Immunohistochemistry Results are summarized in Tables II and III. Nuclear immunoreactivity for both PRA and PRB was detected in ductal epithelial or parenchimal cells, but not in other cell types in all the cases examined (Fig. 1).

There was no correlation between age and IHC score for ERα, PRA, or PRB (data not shown). The IHC score for ERα was significantly lower in high-grade IDC than in PDWA, ADH, low-grade DCIS and low-grade IDC ($P=0.019$, $P<0.001$, $P<0.001$ and $P=0.009$, respectively). The IHC score for PRA in ADH was significantly higher than that of high-grade DCIS and IDC ($P=0.029$ and $P=0.008$, respectively). The IHC score for PRB in high-grade IDC was significantly lower than that of PDWA, ADH and low-grade DCIS ($P=0.042$, $P<0.001$ and $P<0.001$, respectively). Among the DCIS and IDC cases, both PRA and PRB scores were inversely correlated with the histological grades of the lesions (Table III).

Cases in which both PRA and PRB were positive were significantly higher in PDWA and ADH than in IDC and DCIS ($P=0.009$ for PDWA and $P<0.001$ for ADH). The

Table II. Comparison of Immunoreactivity by Histologic Category (Averages Are Shown)

	PDWA (n=27)	ADH (n=27)	DCIS		IDC		*P* value
			Low grade (n=34)	High grade (n=6)	Low grade (n=28)	High grade (n=19)	
Age	44.0	42.8	51.3	56.8	52.7	50.4	$P=0.0069$
ERα IHC score	5.7	6.7	6.4	5.0	5.8	3.3	$P<0.0001$
PRA IHC score	4.3	6.2	4.6	1.8	4.5	2.8	$P=0.0010$
PRB IHC score	4.3	5.6	5.3	1.7	3.3	1.9	$P<0.0001$
Ki67 LI	3.7	4.5	9.5	9.4	21.3	35.9	$P<0.0001$

Table III. Proportion of PR-positive Cases in Each Histological Category

	PDWA	ADH	DCIS			IDC		
			Total	Low	High	Total	Low	High
PRA (+)	85% (23/27)	100% (27/27)	65% (26/40)	68% (23/34)	50% (3/6)	66% (31/47)	79% (22/28)	47% (9/19)
PRB (+)	96% (26/27)	100% (27/27)	75% (30/40)	82% (28/34)	33% (2/6)	55% (26/47)	68% (19/28)	37% (7/19)
PRA (+) PRB (+)	85% (23/27)	100% (27/27)	60% (24/40)	65% (22/34)	33% (2/6)	55% (26/47)	68% (19/28)	37% (7/19)
PRA (–) PRB (–)	4% (1/27)	0% (0/27)	20% (8/40)	15% (5/34)	50% (3/6)	34% (16/47)	21% (6/28)	53% (10/19)

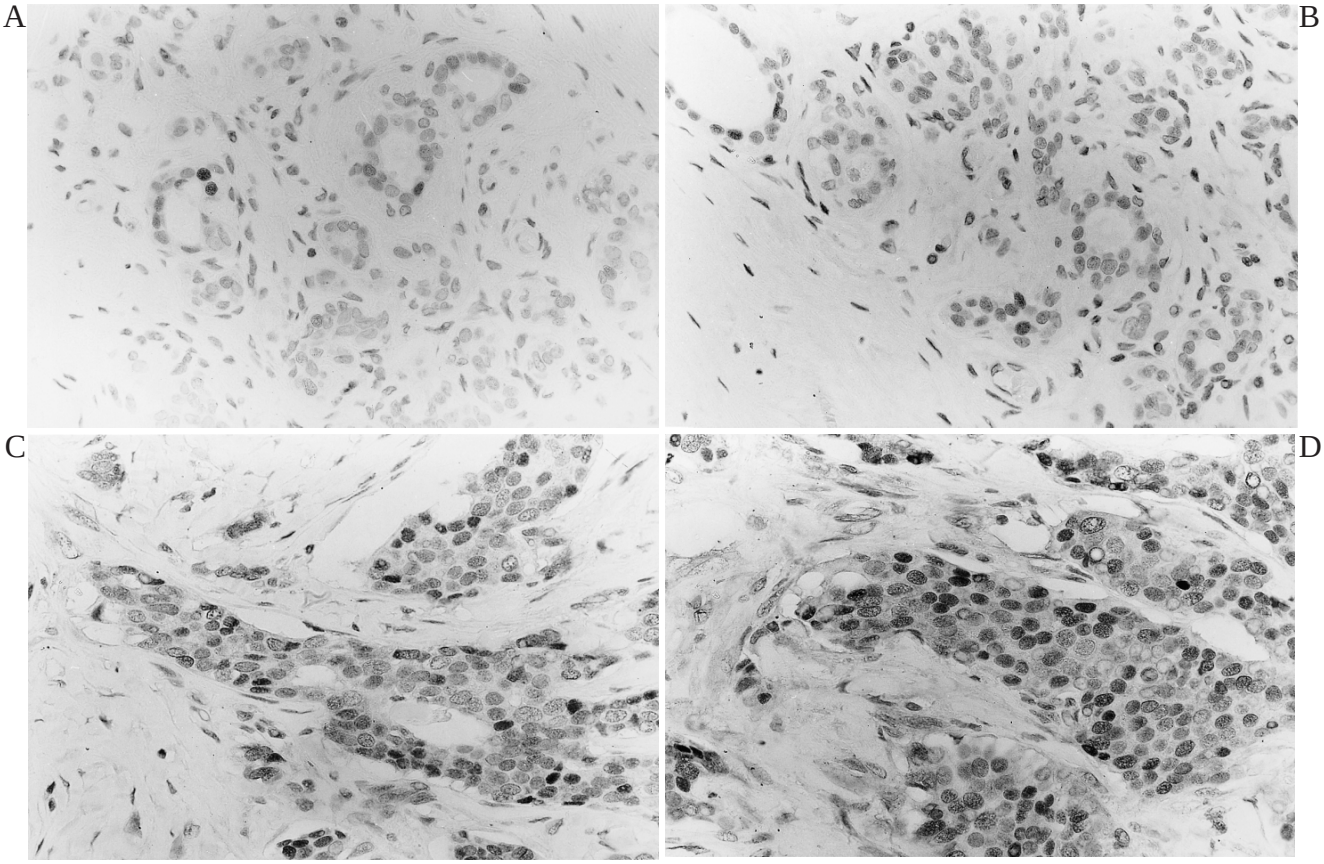

Fig. 1. Immunoreactivity of PRA and PRB in the normal mammary gland (A and B) and invasive ductal carcinoma (C and D). Both PRA (A and C) and PRB (B and D) are stained in the nuclei of ductal epithelia and carcinoma cells (original magnification: 100×).

number of PRA- and PRB-positive cases was also inversely correlated with the histological grade in both DCIS and IDC (P=0.046 for DCIS and P=0.036 for IDC).

In PDWA, ADH, DCIS and IDC, there was a significant positive correlation between PRA and PRB LI (P<0.001, respectively). In PDWA, ADH and DCIS, PRA and PRB were equally distributed in the lesions, but in IDC, PRA tended to be more widely distributed than PRB (Table II).

Correlation betweeen ERα and PR isoforms Results are summarized in Table IV. There was a positive correlation between ERα and PRA IHC score in each histological category examined, but the correlation did not reach statistical significance in PDWA (P=0.078 for PDWA, P=0.004 for ADH, P=0.001 for DCIS and P<0.001 for IDC). On the other hand, the correlation between PRB and ERα IHC score was statistically significant only in ADH and IDC (P=0.002 for ADH and P=0.004 for IDC).

Correlation with clinicopathological parameters Ki67 LI was highest in high-grade IDC and was significantly higher in ERα-negative cases of IDC than in ERα-positive cases of IDC (P=0.033). However, there was no such association in PDWA, ADH or DCIS (data not shown). There were no significant differences in the Ki67 LI between PRA-positive and -negative cases, or between PRB-positive and -negative cases in any of the histological categories examined.

In DCIS, there was no correlation between PRA or PRB status, and the presence of necrosis or architectural pattern, but PRB status was significantly associated with nuclear or histological grade (Van Nuys classification). In

Table IV. Spearman Rank Correlation between ERα and PRA or PRB

	ERα vs. PRA	ERα vs. PRB
PDWA	P=0.0781	P=0.1012
ADH	P=0.0038	P=0.0023
DCIS	P=0.0013	P=0.1643
IDC	P<0.0001	P=0.0036

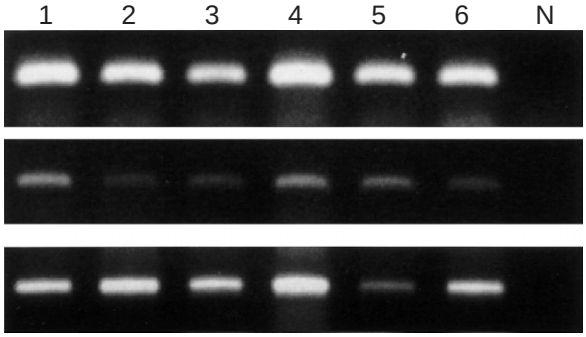

Fig. 2. RT-PCR analysis of total RNA extracted from human breast cancer. Bands of the correct size for PRAB (243 bp) and PRB (429 bp) were detected in all samples. Positive (β-actin) control and negative (N) controls are also shown. Case 2 above is the IDC case as in Fig. 1.

DCIS with high nuclear grades, PRB-positive cases were significantly fewer than in those cases with low nuclear grade (33 to 82%; $P=0.026$; Table III). In IDC, both PRA and PRB IHC scores were significantly associated with histological grade. In high-grade IDC, PRA or PRB-positive cases were fewer than in low-grade IDC (37 to 68%, $P=0.036$; and 47 to 79%, $P=0.027$, respectively; Table III). There were no associations between PRA or PRB IHC scores and lymph node involvement, tumor size, or prognosis of patients in IDC cases (data not shown).

RT-PCR The expression of PRAB and PRB mRNA was detected in all six cases of IDC (Fig. 2). Results of RT-PCR analysis were consistent with those of immunohistochemistry (data not shown).

DISCUSSION

Human PR exists as two isoforms, A and B. Altered ratios of PR isoform expression have been reported to be closely associated with modulations of various progesterone actions,[21–24] but the precise functions of PRA and PRB have not been clearly characterized. In the great majority of progesterone-responsive cells, PRB is a dominant activator of progesterone-responsive target genes, whereas PRA may inhibit this PRB activity. In addition, several investigators have suggested that one PR isoform could modulate the function of the other isoform.[21, 24, 25] PRA, but not PRB, has been demonstrated to inhibit gene transcription induced by other families of steroid receptors, including glucocorticoid, androgen, and mineralocorticoid receptors.[25] This inhibition is not only induced by progestins, but also by some antiprogestins.[25] PRA can also inhibit the transcriptional activity of endogenous ER present in human breast cancer cells.[24] In addition, PRA has been reported to suppress the expression of ERα through the H19 promoter in both hormone-sensitive and hormone-insensitive breast cancer cell lines.[26] These findings suggest the possible inhibitory and/or suppressive nature of PRA in the biological actions of progesterone and/or other steroids. However, Chalbos and Galtier have reported that PRB, but not PRA, inhibits gene transcription induced by ER.[23] In addition, both PRA and PRB, although predominantly PRB, have been reported to be up-regulated by estradiol (E2) but not by tamoxifen or other pure antiestrogens.[19] Therefore, the biological roles or significance of PRA and PRB have yet to be fully characterized in progesterone target tissues.

In the process of human breast cancer development, PDWA is considered to precede ADH, which is also considered a precursor of DCIS. Therefore, it is important to compare the various biological features of these intraductal lesions to gain a better understanding of the pathogenesis in all categories of breast carcinoma. In our study, immunohistochemical scores for both PRA and B isoforms were correlated with ERα in ADH and IDC, but not in PDWA. These findings suggest possible differences of putative estrogen-dependent induction of PR between intraductal epithelial proliferation with and without atypia, but further investigations are required in this regard.

In PDWA and ADH, the distribution of PRA was similar to that of PRB. However, PRA expression was decreased compared to that of PRB in DCIS, which resulted in a lower PRA:PRB ratio. In both DCIS and IDC, PRA and PRB were inversely correlated with histological grades, i.e., both nuclear and architectural differentiation. This finding is consistent with the recent report that PRA expression results in marked changes in the morphology of the cells, especially in the loss of adherent properties,[3] but has no effect on cell proliferation.

Our results also demonstrated that PRA was always equally expressed with PRB in benign proliferative lesions but sometimes dominantly expressed in IDC. These results are consistent with findings of PR isoforms in human breast tumors examined using immunoblot analysis by Graham *et al.*[4] However, Akahira *et al.*[27] recently demonstrated that PRB was dominantly expressed in all types or groups of epithelial ovarian carcinoma, another estrogen-dependent human neoplasm, using both immunohistochemistry and RT-PCR. The biological significance and/or possible significance of PR isoforms in human estrogen-dependent neoplasms requires further investigation for clarification.

In IDC, only PRA, but not PRB, was correlated with ERα. The PRA promoter contains a half-ERE/Sp1 binding site. It has been demonstrated that this half-ERE/Sp1 binding site is protected to a greater extent when MCF-7 cells are treated with estrogen, suggesting that this region may be involved in estrogen-regulated gene expression.[28] These results also suggest that, in human breast cancer, PRA may be more closely regulated by estrogen through

ERα than PRB. Both PRA and B promoters have been reported to be regulated by estrogen, but there may be different pathways, or responsiveness to estrogen actions.[2] Results from our present study suggest that the regulation of PRA by estrogens may differ from that of PRB in invasive ductal carcinoma of the breast.

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