Changes in Expression of Estrogen Receptors α and β in Relation to Progesterone Receptor and pS2 Status in Normal and Malignant Endometrium

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To clarify changes in estrogen receptor (ER) α and ERβ during endometrial tumorigenesis, 48 endometrial carcinomas (endometrioid type), as well as 40 samples of normal endometrial tissue, were investigated using a combination of reverse-transcription and polymerase chain reaction with Southern blot hybridization and western blot assays, and the results were compared with findings for progesterone receptor (PR) and pS2 mRNA status. In addition, 166 carcinomas were also examined for immunohistochemistry, along with 171 normal specimens. Relative amounts of ERα at both mRNA and protein levels were significantly greater than those for ERβ in normal and malignant endometrial lesions. ERα mRNA showed a stepwise decrease from normal or grade (G) 1 through to G3 tumor lesions, in line with changes in the protein levels, in contrast to ERβ mRNA or protein expression, which did not alter, suggesting a shift in the ratio of the two ER subtypes during endometrial tumorigenesis. PR mRNA expression was significantly correlated with ERα, but not ERβ mRNA status. Although significantly higher expression of pS2 mRNA or protein was observed in carcinomas than in the normal cases, there was no apparent association with the ER status. The findings suggest that alteration in estrogen signaling pathways may occur during endometrial tumorigenesis, and provide evidence that ERα expression may play an important role in the regulation of PR, but not pS2 expression in normal and malignant endometrium.

Key words: ERα — ERβ — PR — pS2 endometrial carcinoma

The steroid hormone estrogen plays an important role in the growth, differentiation, and function of many target tissues, in particular in the male and female reproductive systems. In the endometrium, hyperestrogenism is considered to be the major risk factor for development of malignancy, with or without hyperplastic lesions.1,3

Estrogen receptor (ER) α, a member of the superfamily of steroid and thyroid hormone, retinoic acid, and vitamin D receptors, is a ligand-induced transcriptional factor composed of different structural and functional domains.2,3 The transactivation factor domains (TAF-1 and TAF-2) are located within the NH2-terminal (A-B region) and the COOH-terminal (E) sites, whereas the DNA and ligand-binding domains reside in the C and E regions, respectively. Exon 5 deletion splicing of the ER transcript produces a truncated protein which lacks both the TAF-2 and ligand-binding regions, but retains its DNA binding and TAF-1 sequences.9 Previous studies demonstrated that overexpression of the variant is associated with an increase in estradiol-independent growth, resistance to tamoxifen, and enhanced progesterone receptor (PR) expression.4,5

Recently, a novel ERβ subtype has been discovered in human, rat, and mouse tissues, with a high degree of conservation of the DNA-binding domain (96%), and of the ligand-binding domain (58%), as compared to the amino acid sequence of the ERα protein.6 Although changes in the expression of two ER subtypes have been documented during breast and ovarian tumorigenesis,7,8 little is known about the endometrial carcinoma case.

Several estrogen-inducible genes, including PR and pS2, have been described as markers of estrogen responsiveness in human hormone-dependent tumors.4,9 In the present study, we investigated the possible contribution of changes in expression of ERα and ERβ at both mRNA and protein levels during endometrial tumorigenesis. The results were also compared with PR and pS2 status, and several clinicopathological factors.

MATERIALS AND METHODS

Cases A total of 48 endometrial carcinomas (endometrioid type), surgically resected at the Kitasato University Hospital during the period from 1995 to 1998, were investigated for mRNAs. Forty normal endometrial samples (17 cases with a regular menstrual cycle and 23 postmenopausal cases) obtained by total hysterectomy for nonmalignant diseases were also examined. Tissues were snap-frozen immediately after surgical excision and stored at −80°C until use. Histological diagnosis was performed according to the criteria of the International Federation of Gynecology and Obstetrics (FIGO). The carcinoma cases comprised 29 grade (G) 1, 13 G2, and 6 G3 lesions.
Twenty-eight cases were classified as FIGO stage I and 20 as stages II/III/IV, while 22 showed upper myometrial invasion (upper half of myometrium) and 8 were positive for lymph node metastasis.

For immunohistochemical examination, specimens of 166 endometrial carcinomas (endometrioid type; 104 of G1, 35 of G2, and 27 of G3) were obtained by hysterectomy during 1988 to 1998, along with 42 samples of postmenopausal (atrophic) endometrium adjacent to carcinomas and 129 normal biopsy specimens (48 proliferative and 81 secretory stages). These tissues were routinely fixed in 10% formalin and processed for embedding in paraffin wax. Of cases available for investigation of the clinicopathological factors, 110 were categorized in stage I and 20 in stages II/III/IV. In addition, 90 demonstrated upper myometrial invasion and 21 featured metastasis to lymph nodes.

**RT-PCR assay** Total cellular RNAs were extracted using Isogen (Nippon Gene Co., Tokyo) and cDNAs were synthesized from 5 μg of total RNA using RAV-2 reverse transcriptase (TaKaRa, Shiga) in the presence of random primers (TaKaRa) and a ribonuclease inhibitor (TaKaRa). RT-PCR assay was performed with 35 cycles of denaturation at 94°C for 0.5 min, annealing at 55°C for 0.5 min and extension at 72°C for 0.5 min, with a pre-denaturing time of 2 min and a final extension time of 5 min. The primer sequences were: ERα, 5’-CAGGGGGTGAAGTGTTGCTGCTG-3’ and 5’-ATGGGAACCCAGATGATGTACG-3’ [10]; PR, 5’-GCTCCCGCAGCTCGGCTACCC-3’ and 5’-ACAGCTGTGAAGGATCTCATCC-3’ [11]; pS2, 5’-GCAAGAGAAGTGGCGGCCACG-3’ and 5’-TCCGAGTTCAACCCGCCTATCTCAACTAC-3’; and for ERβ mRNA analysis, nested PCR was performed according to the methods described by Vladusic et al. [12] The primers for the first-round PCR were: 5’-TCACTTCTGGCCGTGTCAGCG-3’ and 5’-CCTGGGTTGCCGGCAC-3’ and those for the second-round PCR were: 5’-GGCCAAAGAAGTGGCCGCCACG-3’ and 5’-AAACCTTGAAGTGAGTCCGAGGAG-3’.

Competitive RT-PCR assays for the two ER subtypes were also conducted according to the methods described by Pujol et al., [13] using a common forward primer (5’-AAGAGCTGCCAGCTGCTG-3’) for both genes and different reverse primers for ERα (5’-TGGCAGCTCTCATGTCTCC-3’) and for ERβ (5’-GCCGACACTGGCGGATGCAGGAG-3’).

As a negative control, water was used instead of template cDNA for each examination. To examine the quality and quantity of the synthesized cDNA, the β-actin gene was also amplified as previously described. [14]

**Southern blot hybridization** A 10 μl aliquot of each PCR reaction mixture was electrophoresed in a 3% agarose gel and transferred to a Hybond N nylon membrane (Amersham, Tokyo) with 10× SSC solution overnight. Filters were hybridized overnight with digoxigenin-labeled exon-specific probes using DIG Easy Hyb solution (Boehringer Mannheim, Tokyo). The sequences of the oligonucleotide probes were: probe ERα-1 (5’-AAACGCTCTAA-GAAGAACAGCCTGGGCTTG-3’), probe ERβ (5’-TGA-GCCCCGAGCAGCTAGTGCTACCTCC-3’), probe PR (5’-CCGAGTTCAACCCGCCTATCTCAACTAC-3’), and probe pS2 (5’-GAAAGACAGAATTTGTTTTCCTTGTTGTC-3’). For detection of competitive PCR products, probe ERα-2 (5’-CTCCGCAATTGCTAAGGTGGGAATGATG-3’) and probe ERβ were also applied. Hybridization signals were detected with a DIG Luminescent Detection kit (Boehringer Mannheim). The conditions used for hybridization, washing, and detection were in line with the manufacturer’s recommendations. Hybridization for β-actin gene transcripts was also performed as previously described. [14]

Quantitation of hybridization signals was achieved by densitometric analysis using NIH Image version 1.58 software, according to the method described by Dotzlau et al. [15] Briefly, to control for variations between experiments, a value of 100% was assigned to the case exhibiting the highest signal measured, and all signals were expressed as a percentage of this signal. The relative amounts were calculated by normalization to the hybridization signals for β-actin in each case: the value for mRNA signals of the former was divided by that for the latter.

**Immunohistochemistry** Immunohistochemistry was performed using a combination of microwave-oven heating and the standard streptavidin-biotin-peroxidase complex (LSAB kit; Dako, Copenhagen, Denmark) methods. For detection of ERβ immunoreactivity, biotinylated horse anti-goat IgG (Vector Lab., Burlingame, CA) was used as the secondary antibody. The antibodies used were anti-ERα mouse monoclonal antibody (×80 dilution, Novocastra Lab. Ltd., Newcastle, UK), anti-ERβ (N-19) goat polyclonal antibody (×200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA), anti-PR mouse monoclonal antibody (×80 dilution, Novocastra Lab. Ltd.), and anti-pS2 protein rabbit polyclonal antibody (×1000 dilution, Novocastra Lab. Ltd.).

Scoring of the immunohistochemistry results was carried out on the basis of both the distribution of immunopositive cells and the immunointensity, as previously described. [16, 17] Immunoreactivity scores were generated by multiplication of the values for the two parameters.

**Western blot assay** Tissue samples were homogenized in 0.01 M phosphate-buffered saline (PBS) and the supernatants were used for western blot assays. Twenty-microgram aliquots of proteins were separated by 10% sodium
dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and then electroblotted onto Immobilon-P (Millipore, Tokyo). After blocking, the membranes were incubated overnight at 4°C with optimum dilutions of primary anti-ERα mouse monoclonal antibody (×500 dilution, Novocastra Lab. Ltd.). Binding was visualized using the Western blot Chemiluminescence Reagent (“NEN” Life Science Products, Boston, MA).

Statistics Comparative data were analyzed using the Mann-Whitney U test, the Pearson’s correlation coefficient, and the χ² test. The cut-off for statistical significance was set as P<0.05.

RESULTS

ERα, ERβ, PR, and pS2 mRNA expression Two different sizes of RT-PCR products for ERα transcripts were observed in normal and tumor samples. The larger band (483 bp) and smaller fragment (344 bp) corresponded to the expected sizes for wild type (Wt) and exon 5-deleted (del 5) variants, respectively. The latter was detected only in the presence of the former. Wt ERα transcripts with del 5-variants were detected in 41 (85.4%) of 48 tumors and in all normal tissues. By nested PCR assay, amplicons for ERβ transcripts were also generated as two different fragments, the Wt (429 bp) and del 5-variant (290 bp) forms. In the informative cases, Wt ERβ expression with or without the variant form was observed in 22 (64.7%) of 34 tumors and in 25 (64.1%) of 39 normal cases. The primer sets for PR or pS2 genes produced bands with molecular weights of 174 bp and 246 bp, respectively, positive in 47 (97.9%) and 43 (89.6%) of 48 tumors, and all and 25 (64.1%) of 39 normal samples (Fig. 1).

The relative Wt ERα mRNA values were significantly lower in G3 carcinomas than G1 tumors, while there was no association between Wt ERβ transcripts and histological malignancy. The relative PR mRNA amounts showed a stepwise decrease from G1 through to G3 carcinomas, in contrast to pS2 mRNA values which were significantly higher in G1 or G2 tumors than normal tissue, but without any differences between tumor grades (Fig. 2). No differences in the relative mRNA levels for all transcripts investigated were noted among atrophic, proliferative, and secretory stages in normal endometrium.

Positive correlations between the relative amounts of del 5-variant and Wt forms in both ERs were apparent for both normal and tumor cases (Table I), while no differences in the ratios were noted between the two (data not shown). PR mRNA expression was significantly associated with that of Wt ERα, but not Wt ERβ transcripts, whereas the level of pS2 mRNA was not directly related to any of the hormone receptors (Table I).

By competitive PCR assay, ERα products with a molecular weight of 168 bp were detected in all of the samples investigated, while a 267 bp specific fragment for ERβ

![Fig. 1. mRNA analysis by a combination of RT-PCR and Southern blot hybridization using exon-specific oligonucleotide probes. G, grade; A, atrophic endometrium; P, proliferative phase; S, secretory phase; ER, estrogen receptor; PR, progesterone receptor.](image)
gene was coamplified with 7 (14.6%) of 48 tumors and 3 (8.6%) of 35 normal tissues (Fig. 3).

The relative mRNA amounts of two Wt ERs, PR, and pS2, and ratios of del 5-ER/Wt ERα or β were not related to any of the clinicopathological factors investigated, including FIGO stage, degree of myometrial invasion, or lymph node status, with the exception of an association between high PR mRNA expression and upper myometrial invasion (P<0.05).

**ERα, ERβ, PR, and pS2 protein expression** In normal tissue with the regular menstrual cycle or atrophic endometrium, strong ERα and PR immunoreactivity was detected in glandular, stromal, and myometrial cells, with distinct nuclear staining. Variability in immunointensity and heterogeneous distribution of cells immunopositive for ERα and PR were also noted in carcinoma cases. In contrast, weak ERβ immunoreactivity was sporadically observed in a few cases of normal and tumor epithelial cells. Weak to moderate cytoplasmic pS2 immunoreactivity was sporadically observed in normal or atrophic glandular epithelial cells, although stromal components completely

### Table I. Correlation of mRNA Amounts among ERα, ERβ, PR, and pS2 in Normal and Malignant Endometrium

|                      | Wt ERα      | Wt ERβ      | PR         | pS2        |
|----------------------|-------------|-------------|------------|------------|
|                      | del 5-ERα   | del 5-ERβ   | PR         | pS2        |
|                      | r (P)       | r (P)       | r (P)      | r (P)      |
| Em Ca                | 0.86 (0.0001) | 0.66 (0.0001) | 0.24 (0.09) | 0.25 (0.9) |
|                      | n=50        | n=50        | n=50       | n=50       |
| Normal               | 0.67 (0.0001) | 0.45 (0.004) | 0.2 (0.22) | 0.32 (0.05) |
|                      | n=40        | n=40        | n=40       | n=40       |

ER, estrogen receptor; PR, progesterone receptor; Wt, wild type; del 5, exon 5 deleted variant; r, Pearson’s correlation coefficient; Em Ca, endometrial carcinoma; n, number of cases.

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**Fig. 3.** Competitive PCR assay for ERα and ERβ in endometrial carcinoma cases (lanes, C1 to C14) and normal endometrial tissues (lanes, N1 to N11). Note weak signals for ERβ in C6 and C10 when hybridized with probe ERβ only, but not a combination of probes ERα-2 and ERβ. The short fragment (less than 267 bp) in C5 may be non-specific. Between each hybridization the filter was stripped before being rehybridized with another probe.
lacked any reaction. Moderate to strong immunoreactivity for pS2 protein was observed in some carcinomas, with a variation in immunopositive cell distribution (Fig. 4).

In premenopausal endometrium, average ERα and PR score values showed a significant decrease with transition from proliferative to secretory phases, while no apparent changes in average ERβ and pS2 score values were noted. The values in atrophic endometrium were similar to those of proliferative rather than secretory stages. Average ERα score values significantly decreased in the sequence lead-

Fig. 4. Immunohistochemistry for ERα (A), ERβ (B), and pS2 (C) in endometrial carcinomas. ERα and ERβ show distinct nuclear immunostaining, while cytoplasmic pS2 immunoreactivity is evident. Original magnification, ×200.

Fig. 5. Immunoreactivity scores for the two ER subtypes, PR, and pS2 proteins. A, atrophic endometrium; P, proliferative phase; S, secretory phase; G, grade. The data are mean±SD values. a, \( P<0.0001 \); b, \( P<0.0001 \); c, \( P=0.0268 \); d, \( P<0.0001 \); e, \( P<0.0001 \); f, \( P<0.0001 \); g, \( P=0.0128 \); h, \( P=0.0022 \); i, \( P=0.0028 \); j, \( P<0.0001 \); k, \( P=0.0212 \).
ing from atrophic or proliferative endometrium to G3 carcinomas, while there were no changes in ERβ values among these. PR and pS2 scores also showed a stepwise decrease from G1 through to G3 tumors, although the latter for G1 tumors was significantly higher than for premenopausal endometrial specimens (Fig. 5).

As shown in Table II, ERα but not ERβ values were positively correlated with PR scores in both normal and malignant categories, while pS2 scores did not show any association with the two ERs or the PR status.

The average PR score values were significantly associated with upper myometrial invasion (P=0.0046) and negative lymph node metastasis (P=0.0039), while the ERs and pS2 scores were not related to any of the clinicopathological factors investigated.

**Correlation between mRNA and protein expression for ERα, ERβ, PR, and pS2** Samples were placed in one of three categories, negative, low-, and high-level groups, on the basis of average immunoreactivity scores for each molecule (ERα, 5.4±4.8; ERβ, 0.2±0.7; PR, 6.1±4.4; pS2, 1.6±1.9, mean±SD) in normal and malignant endometrial samples for which mRNA analysis was possible.

High ERα protein scores were related to high levels of Wt ERα mRNA expression and lower values for Wt ERβ, while there was no association between levels of ERβ mRNAs and the proteins. Significant differences in relative amounts of PR or pS2 mRNAs were observed between negative and high-level protein score categories (Fig. 6).

**Western blot assay** Wt ERα proteins were detected as bands with a molecular weight of 65 kDa in both normal and malignant tissues, but 42 kDa bands corresponding to the del 5-ERα variant were not found in any of the samples investigated (Fig. 7A).

ERα positivity in western blot assays was significantly associated with the immunoreactivity scores in all categories (Fig. 7B), with a significant decrease from normal through to G3 tumors (Table III).

**DISCUSSION**

The present study clearly demonstrated the amounts of ERα at both mRNA and protein levels to be significantly greater than those for ERβ in normal and malignant endometrial tissue. Differences in ERβ mRNA positivity between nested and competitive PCR assays may be simply due to their sensitivities. With regard to the absence of differences in mRNA levels among various stages in normal endometrium, in contrast to the immunohistochemical
findings, the possible reasons include the following: 1) the relatively small number of cases investigated for mRNA analysis; 2) the presence of stromal cells expressing ER$\alpha$ or PR.

There are three potential estrogen signaling pathways, involving ER$\alpha$/ER$\alpha$ and ER$\beta$/ER$\beta$ homodimers, as well as ER$\alpha$/ER$\beta$ heterodimers.\(^{18}\) Brandenberger et al.\(^{19}\) have recently demonstrated that endometrial stromal cells isolated from normal endometrium and endometriosis predominantly express ER$\alpha$ mRNA, but ratios of ER$\alpha$:ER$\beta$ mRNAs were significantly higher in the former than the latter, suggesting a difference in the roles of the two ERs in growth responses in normal and ectopic endometrium. In addition, equal or increased ER$\alpha$ mRNA expression in conjunction with a decreased ER$\beta$ mRNA expression indicates differential roles for ER$\alpha$- and ER$\beta$-driven pathways during breast and ovarian tumorigenesis.\(^{7, 8}\)

In our results, Wt ER$\alpha$ mRNA showed a stepwise decrease from normal or G1 through to G3 tumors, in line with changes in the protein expression determined by both immunohistochemistry and western blot assay, in contrast to Wt ER$\beta$ mRNA or protein expression, which did not alter. It is therefore suggested that changes in the ratio of two ER subtypes may cause alteration in estrogen signaling pathways during endometrial tumorigenesis. One reason for the strong ER$\alpha$ mRNA signals in 2 of 6 G3 tumors as compared to the protein levels may be the relatively high sensitivity of PCR assay. In addition, the anomalous results between mRNA and western blot assays for ER$\alpha$, in particular G1 tumors, may be due to the protein degradation during sample preparation and processing or the heterogeneous distribution of tumor cells expressing the protein.

A dominant positive function of del 5-ER$\alpha$ variants has been demonstrated in a yeast transfection assay,\(^{5}\) and these variants may be responsible for estrogen-independent growth and resistance to antiestrogen therapy in breast carcinomas.\(^{4, 20}\) However, not all studies support this hypothesis, and very low transcriptional activity and dominant negative activity when the variant is expressed together with Wt ER$\alpha$ have been reported.\(^{21, 22}\) Ohlsson et al.\(^{23}\) recently found that although the del 5-ER$\alpha$ variant

### Table III. ER$\alpha$ Positivity in Normal and Malignant Endometrium by Western Blot Assay

|                      | Normal endometrium n (%) | Endometrial carcinoma |          |
|----------------------|--------------------------|-----------------------|----------|
|                      | n (%)                    | Grade 1 n (%)         | Grade 2 n (%) | Grade 3 n (%) |
| Positive             | 19 (76%)                 | 11 (45.8%)            | 9 (69.2%)   | 1 (16.7%)     | $P=0.021$ |
| Negative             | 6 (24%)                  | 13 (54.2%)            | 4 (30.8%)   | 5 (83.3%)     |          |

$n$, number of cases.
mRNA is more stable than Wt ERα mRNA, the stability of the variant protein may be significantly lower than that of the Wt form, casting doubt on any biological significance in breast carcinomas. In the present study, although del 5-ERα variant mRNA was frequently coexpressed with the Wt form in both normal and malignant tissues, as well as ERβ mRNA, truncated proteins could not be detected in any of the samples. In addition, the ratios of del 5-variant:Wt ERα mRNA was not associated with any of the prognostic factors investigated. These results suggest that the variant is not of great importance in the development and progression of endometrial carcinomas.

In PR-positive ovarian carcinoma xenografts, reduction of circulating levels of estrogen by ovariectomy has been found to reduce the PR concentration, while exogenous administration causes an increase, supporting the hypothesis that PR levels are regulated by estrogen. In the present case, PR mRNA expression appeared to be paralleled by protein levels, significant correlations for ERα but not ERβ mRNA expression being evident in both normal and tumor cases. Given the induction of PR after 17β-estradiol treatment in the ER-positive PE04 ovarian carcinoma cell line, our data suggest that ERα may play a central role in the regulation of PR expression in the endometrium.

We previously demonstrated that prolonged progesterone administration can suppress cell proliferation in endometrial carcinomas without altering apoptosis, indicating a possible contribution to the regulation of tumor growth. In general, a moderate to high level of PR expression is linked with a good outcome in patients with malignant tumors of the female reproductive system. The significant association between PR expression at mRNA or protein levels and favorable prognostic factors found here may be in line with this.

pS2 protein expression is considered to be an indicator of ER functional activity, since estrogen can promote its transcription, acting via an estrogen response element (ERE) region. In addition to estradiol, epidermal growth factor (EGF), c-H-ras, and c-jun have been reported to regulate pS2 gene expression. Previous studies have demonstrated that it is significantly related to ER protein expression in breast and endometrial carcinomas, so that it may be an additional useful prognostic factor in contrast to the large bowel case where expression is independent of the ER status. In this study, although higher values for pS2 mRNA and protein were observed in endometrial carcinomas than in normal tissues, no association with the ER status or prognostic factors was noted. Although the reason for the discrepancy is unclear, factors other than activated ER may predominantly influence pS2 expression in endometrial tissues. This conclusion is in line with our finding of no cyclic pS2 expression during the normal menstrual cycle.

In conclusion, the present study demonstrated changes in ERα and ERβ mRNA and protein expression between normal and malignant endometrium, and provided evidence that ERα may play an important role in the regulation of PR, but not pS2 expression.

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REFERENCES

1) Deligdisch, L. and Cohen, C. J. Histologic correlates and virulence implications of endometrial carcinoma associated with adenomatous hyperplasia. Cancer, 56, 1452–1455 (1985).
2) Evans, R. M. The steroid and thyroid hormone receptor superfamily. Science, 240, 889–895 (1988).
3) Kumar, V., Green, S., Stack, G., Berry, M., Jin, J. R. and Chambon, P. Functional domains of the human estrogen receptor. Cell, 51, 941–951 (1987).
4) Daffada, A. A. I., Johnston, S. R. D., Smith, I. E., Detre, S., King, N. and Dowsett, M. Exon 5 deletion estrogen receptor messenger RNA expression in relation to tamoxifen resistance and progesterone receptor/pS2 status in human breast cancer. Cancer Res., 55, 288–293 (1995).
5) Fuqua, S. A. W., Fitzgerald, S. D., Chammess, G. C., Tandon, A. K., McDonnell, D. P., Nawaz, Z., O’Malley, B. W. and McGuire, W. L. Variant human breast tumor estrogen receptor with constitutive transcriptional activity. Cancer Res., 51, 105–109 (1991).
6) Mosselman, S., Polman, J. and Dijkema, R. ERβ: identification and characterization of a novel human estrogen receptor. FEBS Lett., 392, 49–53 (1996).
7) Leygue, E., Dotzlaw, H., Watson, P. H. and Murphy, L. C. Altered estrogen receptor α and β messenger RNA expression during human breast tumorigenesis. Cancer Res., 58, 3197–3201 (1998).
8) Brandenberger, A. W., Tee, M. K. and Jaffe, R. B. Estrogen receptor α (ER-α) and β (ER-β) mRNAs in normal ovary, ovarian serous cystadenocarcinoma and ovarian cancer cell lines: down-regulation of ER-β in neoplastic tissues. J. Clin. Endocrinol. Metab., 83, 1025–1028 (1998).
9) Chan, C. M. W. and Dowsett, M. A novel estrogen receptor variant mRNA lacking exons 4 to 6 in breast carcinoma. J. Steroid Biochem. Mol. Biol., 62, 419–430 (1997).
10) Leygue, E. R., Watson, P. H. and Murphy, L. C. Estrogen receptor variants in normal human mammary tissue. J. Natl. Cancer Inst., 88, 284–290 (1996).
11) Carr, M., May, F. E. B., Lennard, T. W. J. and Westley, B. R. Determination of oestrogen responsiveness of breast cancer by competitive reverse transcription-polymerase...
chain reaction. *Br. J. Cancer*, 72, 1427–1434 (1995).
12) Valdusi, E. A., Hornby, A. E., Guerra-Vladusic, F. K. and Lupu, R. Expression of estrogen receptor β messenger RNA variant in breast cancer. *Cancer Res.*, 58, 210–214 (1998).
13) Pujol, P., Rey, J.-M., Nirde, P., Roger, P., Gastaldi, M., Laffargue, F., Rochefort, H. and Maudejonde, T. Differential expression of estrogen receptor-α and -β messenger RNAs as a potential marker of ovarian carcinogenesis. *Cancer Res.*, 58, 5367–5373 (1998).
14) Saegusa, M. and Okayasu, I. Up-regulation of CD44 variant exon expression in endometrial carcinomas: analysis of mRNA and protein isoforms, and relation to clinicopathological factors. *Jpn. J. Cancer Res.*, 89, 291–298 (1998).
15) Dotzlaw, H., Leygue, E., Watson, P. H. and Murphy, L. C. Estrogen receptor-β messenger RNA expression in human breast tumor biopsies: relationship to steroid receptor status and regulation by progestins. *Cancer Res.*, 59, 529–532 (1999).
16) Saegusa, M., Hashimura, M. and Okayasu, I. CD44 expression in normal, hyperplastic, and malignant endometrium. *J. Pathol.*, 184, 297–306 (1998).
17) Sinicrope, F. A., Ruan, S. B., Stephens, L. C., Lee, J. J. and Levin, B. Bcl-2 and p53 oncoprotein expression during colorectal tumorigenesis. *Cancer Res.*, 55, 237–241 (1995).
18) Kuiper, G. G. J. M. and Gustafsson, J.-A. The novel estrogen receptor-β subtype: potential role in the cell- and promoter-specific actions of estrogens and anti-estrogens. *FEBS Lett.*, 410, 87–90 (1997).
19) Brandenberger, A. W., Lebovic, D. I., Tee, M. K., Ryan, I. P., Tseng, J. F., Jaffe, R. B. and Taylor, R. N. Oestrogen receptor (ER)-α and ER-β isoforms in normal endometrial and endometriosis-derived stromal cells. *Mol. Hum. Reprod.*, 5, 651–655 (1999).
20) Castles, C. G., Fuqua, S. A. W., Klotz, D. M. and Hill, S. M. Expression of a constitutively active estrogen receptor variant in the estrogen receptor-negative BT-20 human breast cancer cell line. *Cancer Res.*, 53, 5934–5939 (1993).
21) Rea, D. and Parker, M. G. Effects of an exon 5 variant of the estrogen receptor in MCF-7 breast cancer cells. *Cancer Res.*, 56, 1556–1563 (1996).
22) Desai, A. J., Luqmani, Y. A., Walters, J. E., Coope, R. C., Dagg, B., Gomm, J. J., Pace, P. E., Rees, C. N., Thirunavukkarasu, V., Shousha, S., Groome, N. P., Coombes, R. and Ali, S. Presence of exon 5-deleted oestrogen receptor in human breast cancer: functional analysis and clinical significance. *Br. J. Cancer*, 75, 1173–1184 (1997).
23) Ohlsson, H., Lykkefjeldt, A. E., Madsen, M. W. and Briand, P. The estrogen receptor variant lacking exon 5 has dominant negative activity in the human breast epithelial cell line HMT-3522S1. *Cancer Res.*, 58, 4264–4268 (1998).
24) Langdon, S. P., Gabra, H., Bartlett, J. M. S., Rabiaz, G. J., Hawkins, R. A., Tesdale, A. L., Ritchie, A. A., Miller, W. R. and Smyth, J. F. Functionality of the progesterone receptor in ovarian cancer and its regulation by estrogen. *Clin. Cancer Res.*, 4, 2245–2251 (1998).
25) Langdon, S. P., Hirst, G. L., Miller, E. P., Hawkins, R. A., Tesdale, A. L., Smyth, J. F. and Miller, W. R. The regulation of growth and protein expression by estrogen in vitro: a study of 8 human ovarian carcinoma cell lines. *J. Steroid Biochem. Mol. Biol.*, 50, 131–135 (1994).
26) Saegusa, M. and Okayasu, I. Progesterone therapy for endometrial carcinoma reduces cell proliferation but does not alter apoptosis. *Cancer*, 83, 111–121 (1998).
27) Creasman, W. T., McCarty, K. S., Barton, T. K. and McCarty, K. S., Jr. Clinical correlates of estrogen- and progesterone-binding proteins in human endometrial carcinomas. *Obstet. Gynecol.*, 55, 363–370 (1980).
28) Ehrlich, C. E., Young, P. C. M. and Cleary, R. E. Cytoplasmic progesterone receptors in normal, hyperplastic, and carcinomatous endometrium: therapeutic implications. *Am. J. Obstet. Gynecol.*, 141, 539–546 (1981).
29) Masiakowski, P., Breathnach, R., Bloch, J., Gannon, F., Krust, A. and Chambon, P. Cloning of cDNA sequences of hormone-regulated genes from the MCF-7 human breast cancer cell line. *Nucleic Acids Res.*, 10, 7895–7903 (1982).
30) Brown, A. M., Jeltsch, J. M., Roberts, M. and Chambon, P. Activation of pS2 gene transcription is a primary response to estrogen in the human breast cancer cell line MCF-7. *Proc. Natl. Acad. Sci. USA*, 81, 6344–6348 (1984).
31) Nunez, A. M., Berry, M., Imler, J. and Chambon, P. The 5’ flanking region of the pS2 gene contains a complex enhancer region responsive to oestrogens, epidermal growth factor, a tumour promoter (TPA), the c-h-ras oncoprotein and the c-jun protein. *EMBO J.*, 8, 823–829 (1989).
32) Koshibuya, M., Yoshida, M., Konishi, M., Takemura, M., Yura, Y., Matsushita, K., Hayashi, M. and Tauchi, K. Expression of pS2 protein in endometrial carcinomas: correlation with clinicopathological features and sex steroid receptor status. *Int. J. Cancer*, 74, 237–244 (1997).
33) Thompson, A. M., Elton, R. A., Hawkins, R. A., Chetty, U. and Steel, C. M. PS2 mRNA expression adds prognostic information to node status for 6-year survival in breast cancer. *Br. J. Cancer*, 77, 492–496 (1998).
34) Singh, S., Poulson, R., Hanby, A. M., Rogers, L. A., Wright, N. A., Sheppard, M. C. and Langman, M. J. S. Expression of oestrogen receptor and oestrogen-inducible genes pS2 and ERDS in large bowel mucosa and cancer. *J. Pathol.*, 184, 153–160 (1998).

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