Journal of Pathology
J Pathol 2000; **190**: 554–563.

Original Paper

Up-regulation of pS2 expression during the development of adenocarcinomas but not squamous cell carcinomas of the uterine cervix, independently of expression of c-jun or oestrogen and progesterone receptors

Makoto Saegusa*, Miki Hashimura, Atsuko Hara and Isao Okayasu
Department of Pathology, Kitasato University School of Medicine, 1-15-1 Kitasato, Sagamihara, Kanagawa 228-8555, Japan

*Correspondence to:
Makoto Saegusa, MD,
Department of Pathology,
Kitasato University School of
Medicine, 1-15-1 Kitasato,
Sagamihara, Kanagawa
228-8555, Japan.

Received: 10 May 1999
Revised: 2 August 1999
Accepted: 14 October 1999

Abstract

The pS2 gene product was firstly identified as an oestrogen-induced molecule in a breast cancer cell line, while recent studies demonstrate a close association with mucus-secreting epithelia. To assess pS2 expression in uterine cervical adenocarcinomas (C-ACas) and invasive squamous cell carcinomas (C-ISCCs), a series of 94 and 86 cases, respectively, as well as 77 samples of normal cervix, were immunohistochemically investigated and the results compared with data for expression of oestrogen and progesterone receptors (ER and PR) and c-jun. RT-PCR and western blot assays were also applied to 21 cervical carcinomas and 24 normal tissues. With cervical glandular lesions, significant up-regulation of pS2 expression at both the mRNA and the protein levels was observed for adenocarcinomas *in situ* (AISs) and overt carcinomas, closely linked with mucinous differentiation and tumour grades. pS2 scores were inversely related to ERα status for all cervical glandular categories, while there was no association with ERβ and PR values. In squamous lesions, pS2 values did not differ between normal and malignant lesions, in contrast to the significant down-regulation of ERα expression with tumour development. Although c-jun expression significantly correlated with ERα values for all squamous categories, it did not relate to pS2 status in either C-ACas or ISCCs. These results indicate that alterations in pS2 expression may occur relatively early in the development of cervical glandular, but not squamous lesions, independently of factors known to promote transcription of the pS2 gene. Copyright © 2000 John Wiley & Sons, Ltd.

Keywords: **pS2; ERα; ERβ; PR; c-jun; uterine cervical neoplasms**

Introduction

The uterine cervix is widely accepted to be an oestrogen target organ, the hormone being directly linked with cell proliferation and tissue maturation [1]. Oestrogen has been thought to regulate the transcription of several genes through a single oestrogen receptor (ER) α. For example, the ERα/oestrogen complex can interact with c-jun/c-fos complexes to activate the transcription of target genes through the activator protein 1 (AP-1) enhancer element [2]. Recently, a second ER (ERβ) was identified in humans, rats, and mice [3–5]. In rats, the distribution and relative levels of ERα and ERβ mRNAs are quite different, the former demonstrating moderate to high expression in the uterus, testis, and pituitary, and the latter in the ovary, prostate, and brain [6]. However, little is known about the status of the ER subtypes in the human uterine cervix.

The trefoil (TFF) peptides are a group of small secretory peptides bearing one or more trefoil structural motifs; their expression is closely associated with mucosal defence and reconstitution [7,8]. The pS2 (TFF1) gene encodes an approximately 600 bp mRNA, translated into a cysteine-rich protein containing the trefoil disulphide loop structure which is common to insulin-like growth factors I and II [9,10]. Although pS2 expression has been reported in a variety of human malignancies, the functional significance is not fully understood. In addition, to our knowledge, there have been no reports regarding pS2 expression in normal and malignant uterine cervical lesions.

In the present study, we therefore investigated pS2 expression by immunohistochemistry in normal and malignant glandular and squamous components of the uterine cervix, and compared the results with data for expression of two ER subtypes, the progesterone receptor (PR) and c-jun. A combination of reverse transcription-polymerase chain reaction and Southern blot hybridization (RT-PCR/SBH) for pS2, ERα, ERβ, and PR, and western blot assays for ERα and PR were also performed.

Materials and methods

Cases

A total of 94 cases of uterine cervical adenocarcinoma (C-ACa), surgically resected at the Kitasato University Hospital during the period from 1980 to 1999, were investigated, along with 86 cases of cervical invasive squamous cell carcinoma (C-ISCC) and 77 normal

samples adjacent to carcinoma lesions. These tissues were routinely fixed in 10% formalin and processed for embedding in paraffin wax. Histological diagnoses were performed according to the criteria of the World Health Organization (WHO) and the International Federation of Gynecology and Obstetrics (FIGO). C-ACa cases comprised 42 endometrioid (E-ACa), 36 mucinous (M-ACa), and 16 adenocarcinoma *in situ* (AIS) lesions, while the C-ISCC cases consisted of 33 keratinizing and 54 non-keratinizing types (KT and NKT). Of the 78 M- and E-ACa cases available for the investigation of clinicopathological factors, 50 cases were grade (G) 1, and 28 G2 and G3. In addition, 48 cases were categorized in stage I and 30 in stages II–IV, while 30 demonstrated upper cervical invasion (less than half of cervical depth) and 16 featured metastasis to lymph nodes.

For RT-PCR/SBH and western blot assays, nine C-ACas and 12 C-ISCCs, as well as 24 normal cervical tissues, were snap-frozen immediately after surgical excision and stored at -80°C until use.

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, USA) was used as the secondary antibody. After microwave heat treatment with three 5-min cycles in 10 mM citrate buffer (pH 6.0), sections were incubated overnight at 4°C with optimum dilutions of the primary antibodies as follows: anti-pS2 rabbit polyclonal antibody (pS2p), $\times 1000$ dilution, Novocastra Lab. Ltd., Newcastle, UK; anti-human pS2 mouse monoclonal antibody (pS2m), $\times 50$ dilution, Dako; anti-ERα mouse monoclonal antibody, $\times 100$ dilution, Novocastra Lab. Ltd.; anti-ERβ (N-19) goat polyclonal antibody, $\times 200$ dilution, Santa Cruz Biotechnology, Santa Cruz, USA; anti-PR mouse monoclonal antibody, $\times 100$ dilution, Novocastra Lab. Ltd.; and anti-c-jun (KM-1) mouse monoclonal antibody, $\times 100$ dilution, Santa Cruz Biotechnology.

Scoring of the immunohistochemistry results was performed on the basis of both the distribution of immunopositive cells and the immuno-intensity, as previously described [11,12]. Briefly, based on the percentages of immunopositive epithelial cells in the totals of normal or neoplastic cells, subdivision was into five categories as follows: 0, all negative; 1, $<10\%$ positive cells; 2, 10–30%; 3, 30–50%; and 4, $>50\%$. The immuno-intensity was also subclassified into four groups: 0, negative; 1+, weak; 2+, moderate; and 3+, strong. Immunoreactivity scores were generated by multiplication of the values for the two parameters. As positive controls, normal cervical components were used for ERα and PR, ovarian cystadenoma cases for ERβ, and breast carcinoma case for c-jun and pS2.

RT-PCR

Total cellular RNAs were extracted using Isogen (Nippon Gene Co., Tokyo, Japan) and cDNAs were synthesized from 5 µg of total RNA using RAV-2 reverse transcriptase (Takara, Shiga, Japan) in the presence of random primers (Takara) in a 20 µl reaction volume at 42°C for 60 min.

One microlitre of cDNA solution was amplified by Taq polymerase (Takara) in a volume of 10 µl. For mRNA detection of pS2, ERα, and PR genes, the PCR procedure 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 predenaturing time of 2 min and a final extension time of 5 min. The primer sequences were pS2, 5′-ACCATGGAGAACAAGGTGAT-3′ (sense, nucleotides 47–66) and 5′-AAATTCACACTCCTCTTCTG-3′ (antisense, nucleotides 273–292); ERα, 5′-CAGGGGTGAAGTGGGGTCTGCTG-3′ (sense, nucleotides 1060–1082 in exon 4) and 5′-ATGCGGAACCGAGATGATGTAGC-3′ (antisense, nucleotides 1520–1542 in exon 6) [13]; PR, 5′-GCTCCCGCAGCTCGGCTACC-3′ (sense, nucleotides 2285–2304) and 5′-ACAGCCTGATGCTTCATCCC-3′ (antisense, nucleotides 2454–2473) [14]. For ERβ mRNA analysis, nested PCR was performed according to the methods described by Vladusic *et al.* [15]. The primers for the first-round PCR were 5′-TCACTTCTGCGCTGTCTGCAGCG-3′ (sense, nucleotides 297–319) and 5′-CCTGGGTCGCTGTGACCAGA-3′ (antisense, nucleotides 1095–1114). The primers for second-round PCR were 5′-GGCCAAGAGAAGTGGCGGCCACG-3′ (sense, nucleotides 592–613) and 5′-AAACCTTGAAGTAGTTGCCAGGAG-3′ (antisense, nucleotides 996–1020).

Competitive RT-PCR assays for the two ER genes were also carried out according to the methods described by Pujol *et al,* [16], using a common forward primer (5′-AAGAGCTGCCAGGCCTGCC-3′) for both subtypes and two specific reverse primers (5′-TTGGCAGCTCTCATGTCTCC-3′ for ERα and 5′-GCGCACTGGGGCGGCTGATCA-3′ for ERα, respectively).

As a negative control, water was used instead of template cDNA for each examination. To assess the quality and quantity of the synthesized cDNA, the β-actin gene was also amplified as previously described [17].

SBH

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, Japan). Filters were hybridized overnight with digoxigenin-labelled exon-specific oligonucleotide probes with sequences as follows: probe pS2 (5′-GAAAGACAGAATTGTGGTTTTCCTGGTGTC-3′, nucleotides 149–178), probe ERα-1 (5′-AAACGCTCTAAGAAGAACAGCCTGGCCTTG-3′, nucleotides 1217–1246 in exon 4), probe ERβ (5′-TGAGCCCCGAGCAGC-

TAGTGCTCACCCTCC-3′, nucleotides 648–677 in exon 4), and probe PR (5′-CCGCAGGTCTACCCG-CCCTATCTCAACTAC-3′, nucleotides 2346–2375). For detection of competitive PCR products, probe ERα-2 (5′-CTCCGCAAATGCTACGAAGTGGGA-ATGATG-3′) and probe ERβ were 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 [17].

Quantitation of hybridization signals was achieved by densitometric analysis using NIH Image version 1.58 software, according to the method described by Dotzlaw *et al.* [18]. Briefly, to control for variations between experiments, a value of 100% was assigned to the case exhibiting the highest signal measured and all other signals were expressed as a percentage of this. The relative amounts of pS2, ERα, ERβ, and PR were calculated by normalization to the hybridization signals for β-actin in each case: the values for mRNA signals for the former were divided by that for the latter.

Western blot assay

Tissue samples were homogenized in 0.01 M phosphate-buffered saline (PBS) and the supernatants were used for western blot assays. Thirty micrograms of proteins was separated by 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and then electroblotted onto immobilon-P (Millipore, Tokyo, Japan). After blocking, the membranes were incubated overnight at 4°C with optimum dilutions of primary anti-ERα (×500 dilution, Novocastra Lab. Ltd.) and anti-PR (×500 dilution, Novocastra Lab. Ltd) mouse monoclonal antibodies. Reactivity was visualized using the western blot Chemiluminescence Reagent (NEN™ Life Science Products, Boston, USA). Normal endometrial tissues were used as positive controls for both proteins.

Statistics

Comparative data were analysed using the Mann–Whitney *U* test and Pearson's correlation coefficient. The cut-off for statistical significance was set as $P < 0.05$.

Results

pS2, ERα, ERβ, PR, and c-jun expression in cervical glandular lesions

Negative or weak immunoreactivity with both monoclonal and polyclonal pS2 antibodies was observed in cervical glandular epithelial cells, whereas stromal tissues completely lacked any immunoreaction. ERα and PR strongly bound to glandular and stromal elements, in contrast to ERβ, which was completely negative. The antibody against c-jun occasionally demonstrated some binding to glandular epithelial components.

Intense and abundant cytoplasmic pS2 immunoreactivity was present in AIS and C-ACa lesions, in particular the mucinous type, when the polyclonal antibody was applied. Although the monoclonal pS2 antibody also reacted with the luminal surface, with or without cytoplasmic staining, the immunopositive areas were more limited. Weak to moderate immunoreactivity for the two ER subtypes, PR, and c-jun was sporadically observed (Figures 1, 2, and 4E).

The average values for both pS2m and pS2p scores were significantly higher in C-ACas than in normal

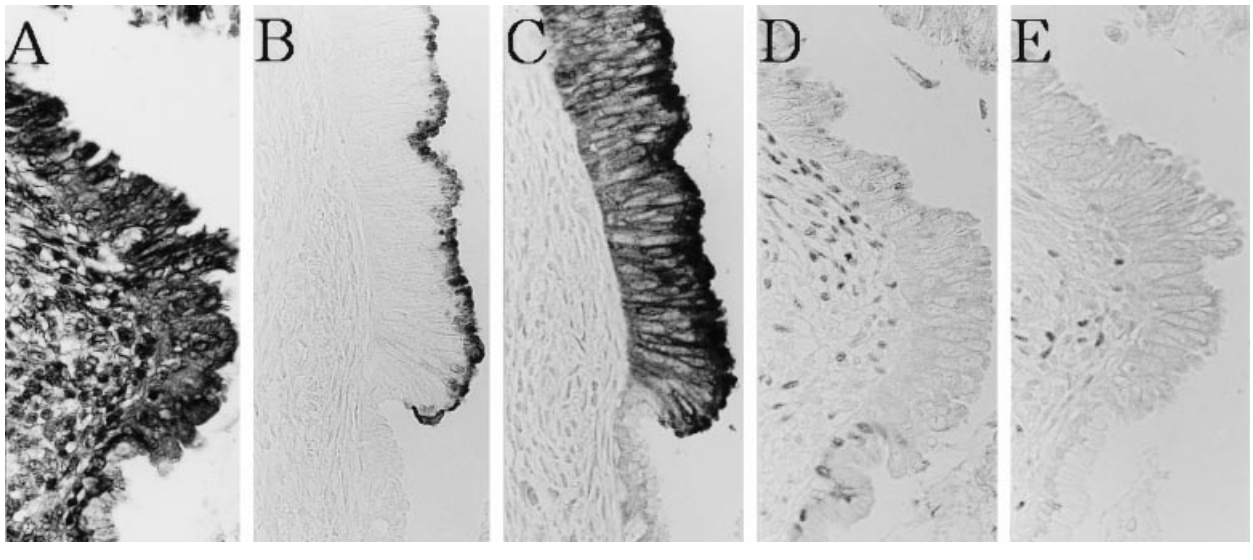

Figure 1. Semiserial sections of an AIS lesion. (A) H&E staining. (B, C) Monoclonal pS2 antibody reaction is mainly at the luminal surface (B), while strong cytoplasmic staining is also evident with the polyclonal antibody (C). Note the negative immunoreaction of normal cervical glandular epithelial and stromal elements. (D) ERα immunoreactivity is apparent in normal cervical glandular and stromal components, while the AIS lesion completely lacks any immunoreaction. (E) PR immunoreactivity is limited to stromal elements. ×400, reduced to 80% in printing

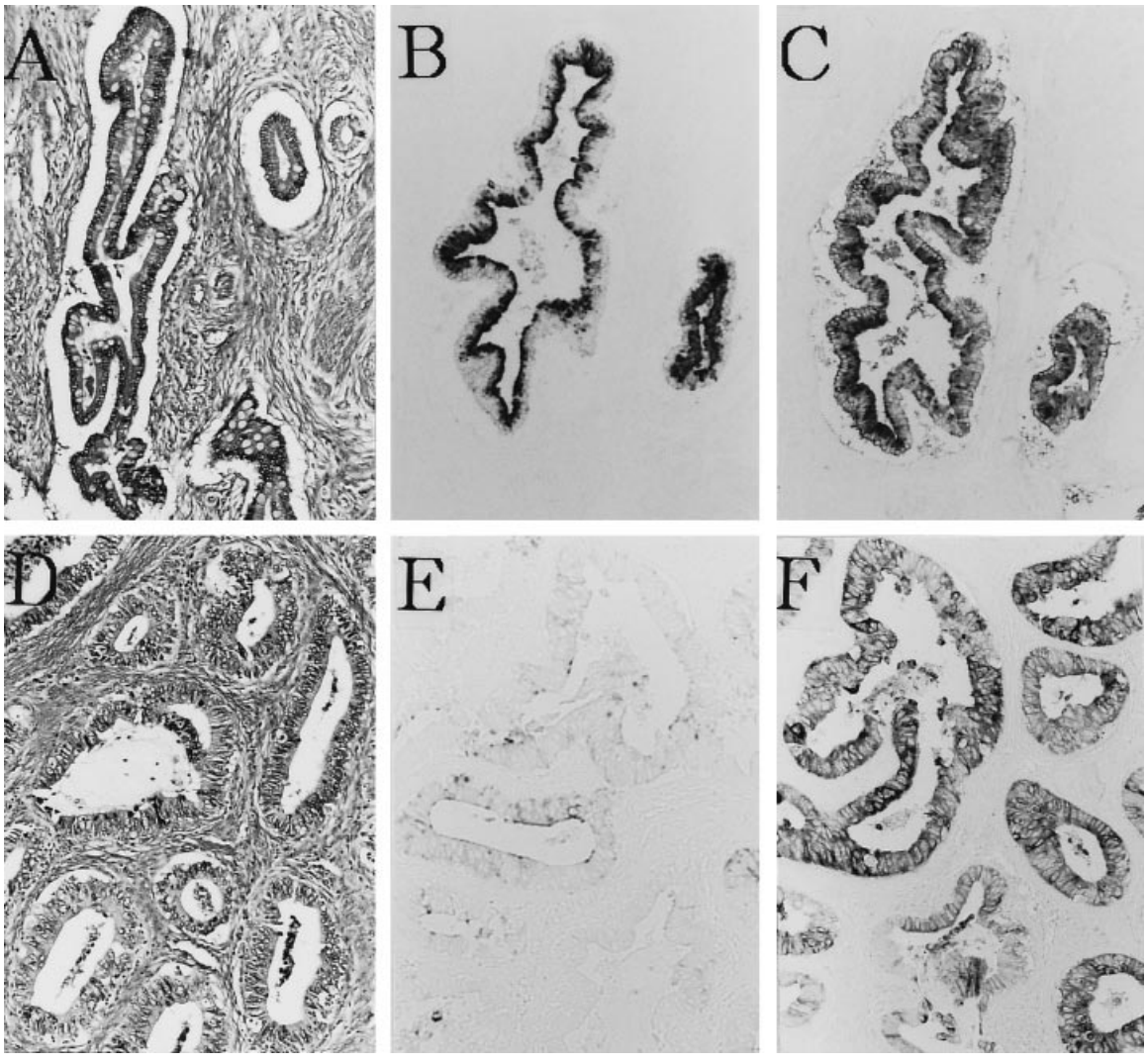

Figure 2. Semiserial sections of mucinous (A–C) and endometrioid (D–F) adenocarcinomas. (A, D) H&E staining. (B, E) pS2 monoclonal antibody binding is strong at the luminal surface of the mucinous lesion. (C, F) Note the strong cytoplasmic immunoreactivity with the polyclonal pS2 antibody. × 100

glands, while the average ERα and PR scores were significantly lower in the former. In ACas, there were significant differences in pS2 but not hormone receptor status among histological phenotypes. No alteration in c-jun scores was observed between normal and tumour tissues (Figure 3).pS2m scores were significantly higher ($p=0.049$) in G1 C-ACas than in G2/G3 tumours, while c-jun values were significantly lower ($p=0.026$). Values for the two ERs or PR were not linked to any of the several clinicopathological factors investigated.

pS2m scores positively correlated with pS2p values ($r=0.79$, $p<0.0001$) but were inversely linked with ERα status ($r=-0.41$, $p<0.0001$) for all cervical glandular categories, while there was no association with ERβ, PR, or c-jun values.

pS2, ERα, ERβ, PR, and c-jun expression in cervical squamous lesions

Strong immunoreactivity for ERα and c-jun was frequently observed in normal squamous epithelia, in contrast to pS2m, ERβ, and PR. In C-ISCC lesions, weak immunoreactivity for pS2m, the two ERs, and PR was sporadically present in a few tumour cells, while c-jun-positive clusters were occasionally found in tumour nests, in particular in KT-ISCCs (Figures 4A–D).

Significantly decreased ERα scores were observed in malignant lesions compared with the normal case, while there were no differences in pS2m, ERβ, and PR values. The average c-jun expression decreased in the sequence leading from normal to NKT-ISCCs, this being significant (Figure 5).

pS2m scores did not relate to the status for the two ERs, PR or c-jun in any of the squamous lesions, although a significant correlation ($r=0.5$, $p<0.0001$) between ERα and c-jun values was noted for all categories.

RT-PCR assay

Amplicons of the β-actin gene could be successfully amplified from the RNAs obtained from all samples, with a molecular weight of 446 bp. ERα and PR

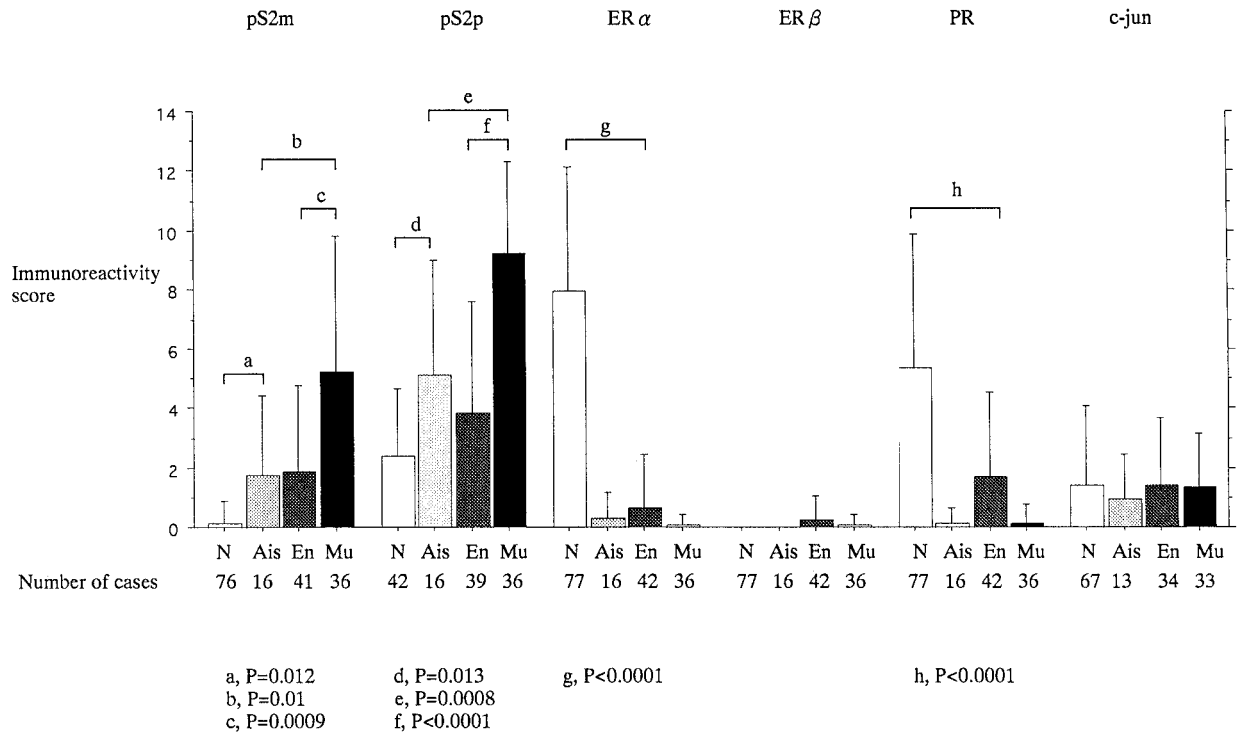

Figure 3. Immunoreactivity scores for pS2, ER, PR, and c-jun in cervical glandular lesions. N = normal cervical glandular epithelium; Ais = adenocarcinoma *in situ*; En = endometrioid adenocarcinoma; Mu = mucinous adenocarcinoma. The data are mean ± SD values

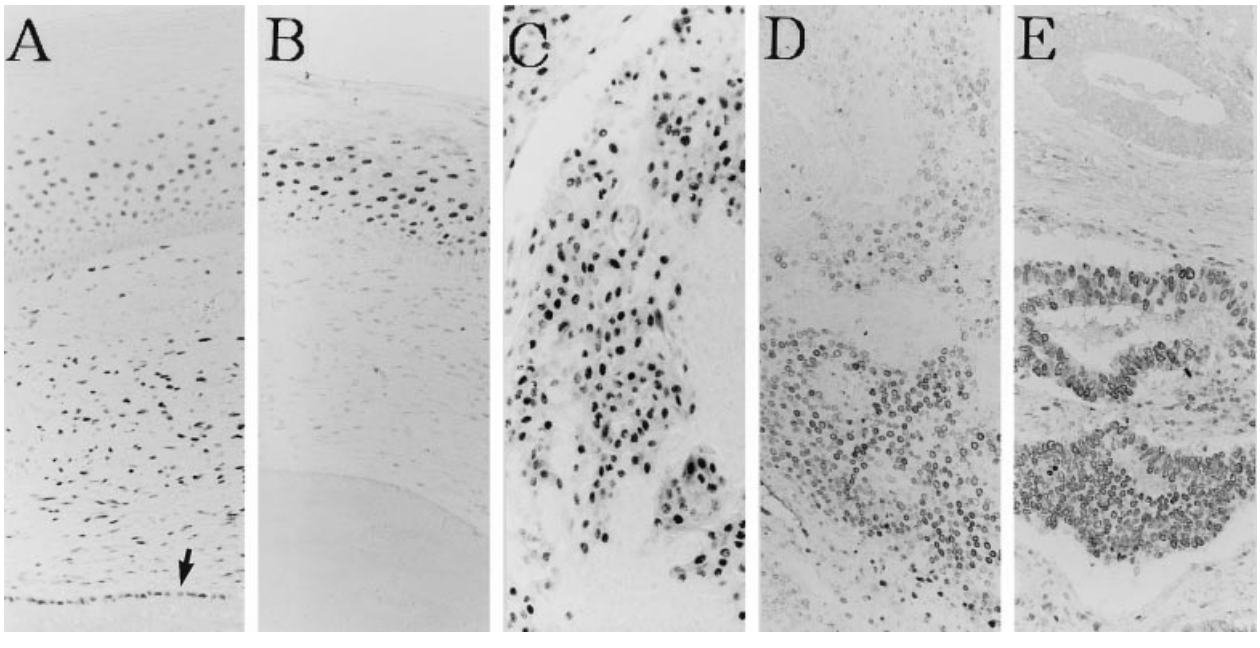

Figure 4. (A, B) Semiserial sections of normal cervix. ERα immunoreactivity (A) is observed in squamous, stromal, and glandular (indicated by arrow) elements, but c-jun immunoreactivity (B) is limited to the squamous epithelium. (C) Note the c-jun immunoreactivity in a keratinizing type squamous cell carcinoma. (D, E) Strong ERβ immunoreactivity in a keratinizing type squamous cell carcinoma (D) and an endometrioid adenocarcinoma (E). × 200, reduced to 80% in printing

immunopositive stromal cells were rarely observed in sections adjacent to lesions taken for mRNA analysis (Table 1).

With primer sets corresponding to the whole translated region of the pS2 gene, amplicons with a molecular weight of 246 bp were observed for all C-ACas, 10 (83.3%) of 12 C-ISCCs, and 16 (66.7%) of 24 normal tissues. Two different sizes of RT-PCR products for ERα transcripts were observed, the larger band (483 bp) and smaller fragment (344 bp) corresponding to the expected sizes for wild-type (wt) and exon 5-deleted (del 5) variant forms, respectively.

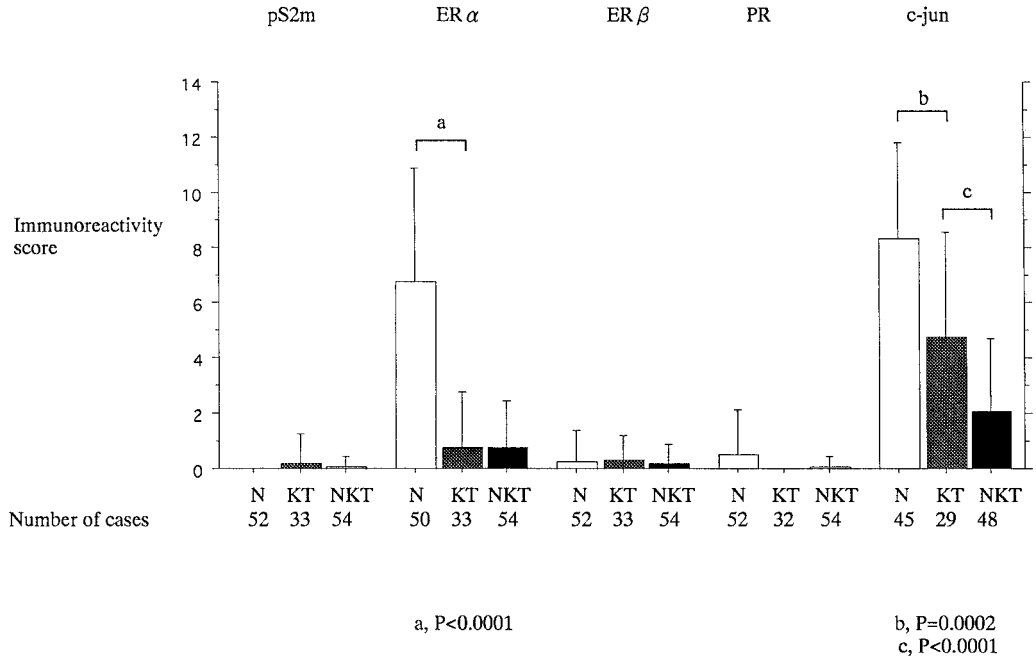

Figure 5. Immunoreactivity scores for pS2, ER, PR, and c-jun in cervical squamous lesions. N = normal squamous epithelium; KT = keratinizing type squamous cell carcinoma; NKT = non-keratinizing type squamous cell carcinoma. The data are mean ± SD values

Table 1. Summary of the RT-PCR/SBH, immunohistochemical, and western blot assays for cervical tumours

		Relative intensity (/β-actin)					Immunoreactivity score					Western		Stroma/tumour*	
No.	**Histology**	**pS2**	**wt ERα**	**del 5/ERα**	**wt ERβ**	**PR**	**pS2m**	**pS2p**	**ERα**	**PR**	**c-jun**	**ERα**	**PR**	**ERα**	**PR**
A1	E-ACa, G1	0.73	1.27	0.37	0	0.9	0	2	2	6	2	N	N	<10%	<10%
A2	M-ACa, G3	1.27	1.03	0.32	0	0	0	9	0	0	2	P	N	<10%	<10%
A3	E-ACa, G1	1.3	1.3	0.41	0	0	3	6	2	2	2	ND	ND	<10%	<10%
A4	M-ACa, G3	1.53	0.57	0.18	0	0	9	12	0	0	ND	ND	ND	0%	0%
A5	E-ACa, G3	0.27	1.33	0.33	0	0.73	ND	ND	ND	ND	ND	ND	ND	0%	0%
A6	E-ACa, G3	0.63	1.4	0.26	0	0.93	0	0	0	9	6	P	P	0%	0%
A7	E-ACa, G3	0.53	1.3	0.46	1.71	0.43	0	ND	2	4	4	ND	ND	10%	10%
A8	E-ACa, G2	0.17	1.13	0.32	0	0.17	4	ND	9	6	9	P	N	30%	10%
A9	E-ACa, G1	0.67	0.43	0.38	0	0.33	0	3	0	0	0	P	P	0%	0%
S1	SCC, NKT	0.17	1.33	0.1	0	1.1	ND	ND	ND	ND	ND	ND	ND	0%	0%
S2	SCC, NKT	0.4	0.87	0.19	0	0.37	0	ND	0	0	2	N	N	0%	0%
S3	SCC, KT	0	0.53	0.12	0	0	0	ND	0	0	4	N	N	0%	0%
S4	SCC, NKT	0.07	0.73	0.32	0	0	0	ND	0	0	3	N	N	<10%	<10%
S5	SCC, KT	0.17	0.57	0.29	0	0.03	0	0	0	0	9	N	N	0%	0%
S6	SCC, NKT	0.57	0.6	0.33	0	0	0	0	0	0	4	N	N	<10%	0%
S7	SCC, NKT	0.57	0.33	0.6	0	0	0	0	0	0	4	P	P	<10%	<10%
S8	SCC, NKT	0.73	0.37	0.27	0	0	0	0	0	0	3	N	N	<10%	<10%
S9	SCC, NKT	0.27	0.33	0.1	0.32	0.02	0	0	0	0	6	N	N	0%	<10%
S10	SCC, NKT	1	0.67	0.35	0	0	0	0	0	0	6	N	N	0%	0%
S11	SCC, NT	0	0.03	0	0	0	0	0	0	0	0	N	N	0%	0%
S12	SCC, KT	0.73	0.03	0	0	0	0	0	0	0	6	N	N	0%	<10%
Normal cervix (*n* = 24)	0.29 ±0.34	0.89 ±0.2	0.31 ±0.1	0	0.49 ±0.56										

E-ACa = endometrioid adenocarcinoma; M-ACa = mucinous adenocarcinoma; SCC = squamous cell carcinoma; NKT = non-keratinizing type; KT = keratinizing type; wt ERα = wild type oestrogen receptor α; del 5/ERα = proportion of exon 5-deleted variants : wild type oestrogen receptor α; PR = progesterone receptor; pS2m = pS2 monoclonal antibody; pS2p = pS2 polyclonal antibody; ND = not done; N = negative; P = positive.
*Proportion of ER or PR immunopositive non-neoplastic stromal cells relative to tumour area in sections adjacent to lesions for mRNA analysis.

The latter was detected only in the presence of the former. Wt ERα transcripts with del 5 variants were detected in all C-ACa and normal samples and in 10 (83.3%) of 12 C-ISCCs. In contrast, amplicons for ERβ transcripts were observed in only one C-ACa, two C-ISCCs, and one normal sample, the two different fragments corresponding to the wt (429 bp) and del 5 variant (290 bp) forms. The primer sets for the PR

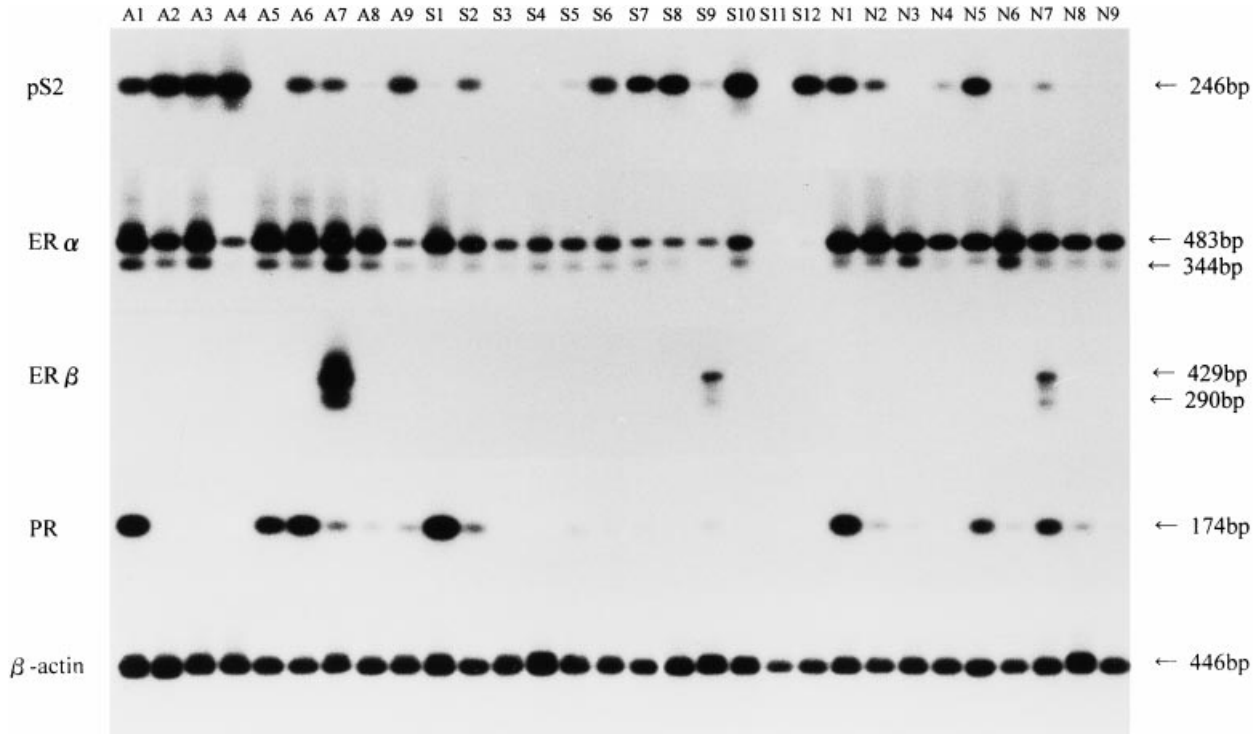

Figure 6. mRNA analysis by a combination of RT-PCR and SBH assays. Case numbers correspond to those in Table I

gene produced a single band with a molecular weight of 174 bp, with 6 (66.7%) of 9 ACas, 3 (25%) of 12 C-ISCCs, and 21 (87.5%) of 24 normal tissues (Figure 6).

Data for relative intensity obtained with RT-PCR/SBH assays are summarized in Table 1. The mRNA expression of pS2 and wt ERα in C-ACas was significantly higher than in either normal or C-ISCC tissues, while PR mRNA levels were decreased from normal through to C-ISCC lesions, this being significant. There were no differences in the proportions of del 5-ERα:wt ERα among the three groups (Figure 7). A positive correlation ($r=0.42$, $p=0.001$) for all categories between mRNA levels of ERα and PR was

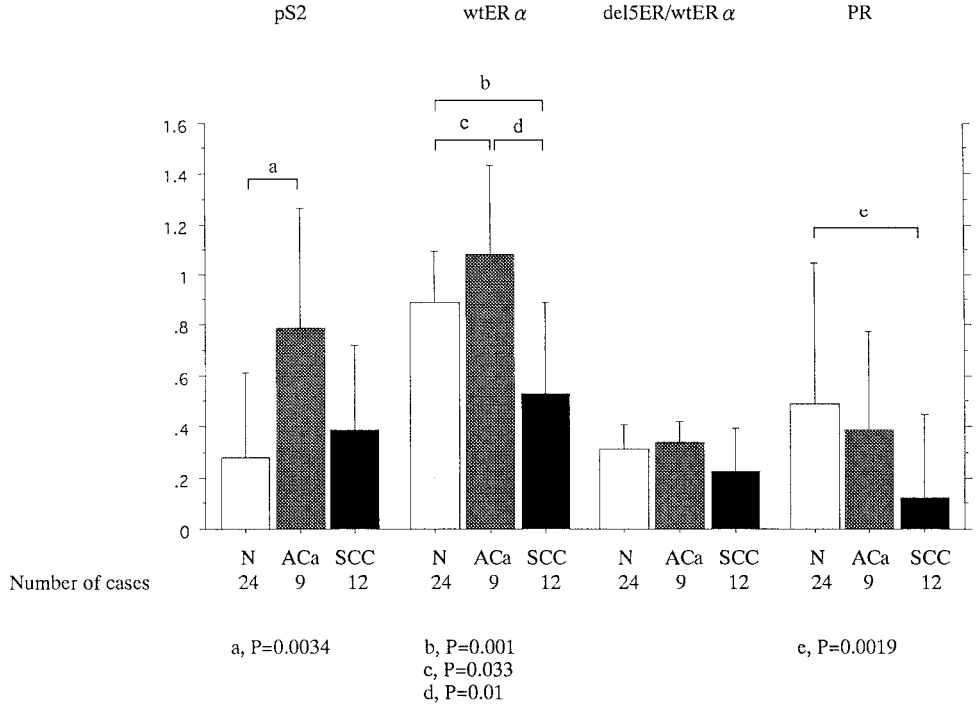

Figure 7. Relative amounts of mRNA expression in normal and malignant cervical lesions. wt ERα = wild type oestrogen receptor α; del 5/ERα = proportion of exon 5-deleted variants : wild type oestrogen receptor α; N = normal cervical tissues including glandular and squamous epithelial and stromal components; ACa = adenocarcinoma; SCC = squamous cell carcinoma. The data are mean ± SD values

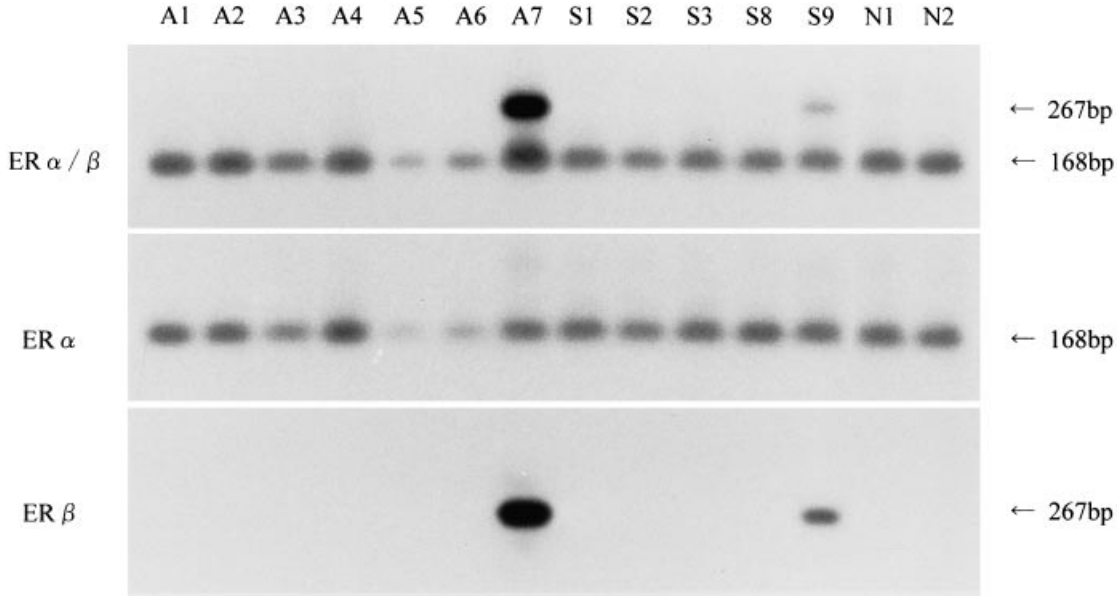

Figure 8. Competitive RT-PCR assay for ERα and ERβ genes. Case numbers correspond to those in Table I

noted, while pS2 mRNA values were not related to the status of ERα and PR mRNAs.

The hybridization signal intensities for pS2 and PR correlated significantly with their immunoreactivity scores (pS2m, $r=0.49$, $p=0.035$; pS2p, $r=0.8$, $p=0.0006$; PR, $r=0.82$, $p<0.0001$). Although ERα also showed a similar tendency, this did not reach significance ($r=0.45$, $p=0.055$).

In the competitive PCR assay, co-amplification products for ERα and ERβ mRNAs were a 168 bp and a 267 bp specific fragment, respectively. ERβ products were detected in two cases of C-ACa, two of C-ISCC, and two of normal tissues, while ERα mRNAs were amplified with all samples investigated. The ratios of ERα:ERβ did not vary among the three groups (Figure 8).

Western blot assay

wt ERα proteins with a molecular weight of 65 kD were detected in 4 of 5 ACas, 1 of 12 SCCs, and all 24 normal samples, along with approximately 50 kD fragments. In contrast, a 42 kD band corresponding to the del 5-ERα proteins was not found in any cases.

PR proteins in an approximately 110 kD band were found in 2 of 5 ACas, 1 of 12 SCCs, and 5 of 24

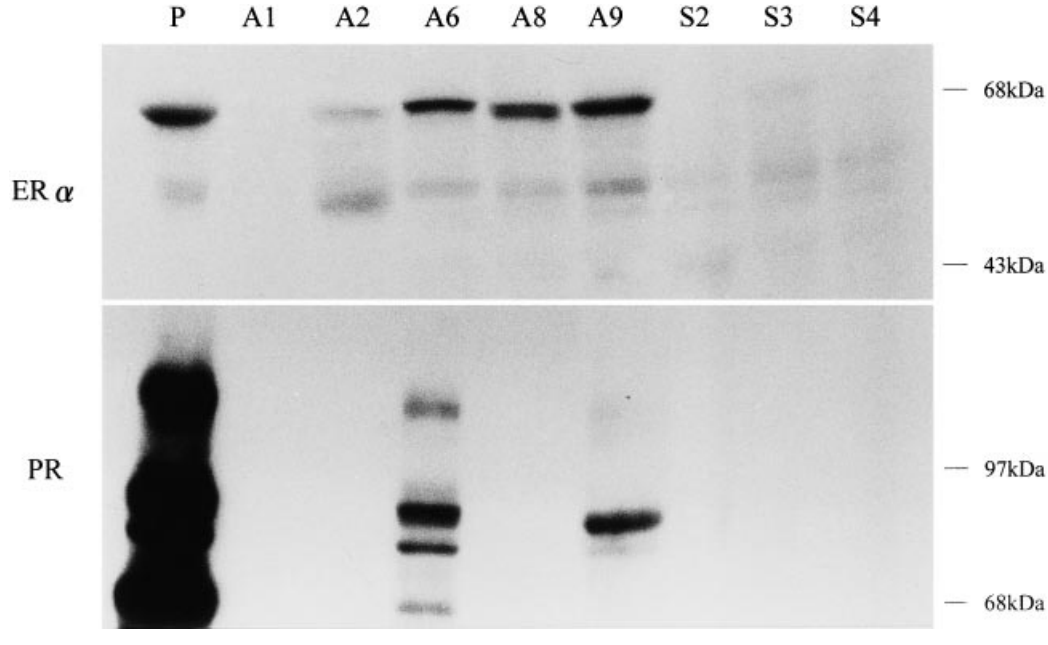

Figure 9. Western blot assay for ERα and PR. Case numbers correspond to those in Table I. P = positive control

normal tissues, along with several smaller bands, the latter possibly being N-terminally truncated forms (Figure 9).

Discussion

pS2 expression has been demonstrated to be increased by oestrogenic stimulation at the level of transcription in breast cancer cell lines, indicating that pS2 is a member of the oestrogen-inducible gene family [9,19]. In the present immunohistochemical study, however, negative or low levels of pS2 expression were evident in normal cervical glandular and squamous components, despite relatively high ERα expression at both mRNA and protein levels. Similar disconcordance has also been reported for normal colorectal tissues [20,21], suggesting a lack of any direct correlation.

A progressive increase of pS2 expression from normal through to hyperplastic or malignant lesions has been demonstrated in breast and endometrial tissues [22,23]. The finding in this study of pS2 gene up-regulation in C-ACas, including AIS lesions, is thus of interest. The fact that M-ACas rather than E-ACas were particularly affected suggests that alterations in pS2 expression may occur as a relatively early event in the development of C-ACas, closely linked with mucinous differentiation. Our demonstration of no equivalent change in squamous lesions points to dysregulation dependent on the cell type.

In cells expressing only the ERα or ERβ subtypes, homodimers of either can interact with oestrogen response elements, while heterodimers can also be formed in cells expressing both ERs, in line with the ratios of the subtypes [24]. Co-expression of ERα and ERβ has been reported in breast and ovarian tissues, with a close linkage between changes in the ratios and tumourigenesis [25]. In this study, ERα predominantly expressed in terms of both mRNA and protein levels, compared with ERβ in normal and neoplastic cervical tissues. However, the loss or down-regulation of ERα protein expression was also evident in both C-ACa and C-ISCC lesions, in line with previous studies [26,27].

With regard to correlations among pS2, ERs, PR, and c-jun, ERα showed an inverse association with pS2 expression in cervical glandular lesions, although a positive link with PR status was evident. Given that c-jun/c-fos complexes can interact with the AP-1-like sequence within the 5′-regulatory region of the pS2 gene [28], it might be expected that c-jun would contribute to the regulation of pS2 transcription. Our results, however, revealed no association between c-jun and pS2 expression, suggesting that other factors are more important in this respect. In contrast, a positive association between ERα and c-jun expression was noted in squamous lesions, in line with the finding that ER is able to bind to the AP-1 response element via c-jun and c-fos complexes [29]. In addition, it appeared that changes in c-jun expression may play an important role in tumour development and differentiation of C-ISCCs.

The clinical significance of pS2 expression appears to depend on the variety of human malignancy. In breast and endometrial carcinomas, pS2 expression has been considered a favourable prognostic factor, being positively linked with ER expression [23,30], while in gastric, colorectal, and ovarian carcinomas it is associated with tumour aggressiveness, independently of ER status [20,21,31,32]. In our series, pS2 expression was not associated with clinicopathological factors for C-ACas, with the exception of tumour differentiation. One explanation for the discrepant results might be the existence of differences in pS2 transcriptional pathways.

Alternative splicing of ER mRNAs generates several variant forms, featuring, for example, exon 2, 3, 5, or 7 deletions [13]. The del 5-ER mRNA translates to a protein truncated in the NH_2-terminal part of the hormone-binding domain, showing a constitutively active transcriptional activator in a yeast transfection assay [33], Castles *et al.* [34] have reported significant amounts of del 5-ER mRNA in a breast cancer cell line (MCF7), although they were unable to immunoprecipitate any variant proteins. Ohlsson *et al.* [35] demonstrated that del 5-ER protein expression may not be correlated with mRNA expression, suggesting that the variant protein is short-lived. These observations are in line with our findings. In addition, the lack of any difference in the proportions of the del 5-ERα type between normal and tumour lesions suggests that this variant may not have a major role in cervical tumourigenesis.

With RT-PCR and western blot assays, the presence of non-epithelial components expressing ER and PR proteins may cause anomalous results. In this study using frozen samples, although a few stromal elements with ERα or PR were presumably included in tumour tissues, the relative mRNA intensities for pS2, ERα, and PR appeared to be correlated with the immunoreactivity scores. The contrasting lack of association between mRNA and western blot assays for ERα and PR in tumour samples may have been due to the greater sensitivity of the RT-PCR approach. Similar findings have also been reported by several investigators [36–38].

In conclusion, the present study demonstrated that alteration in pS2 expression is closely associated with glandular, but not squamous tumourigenesis of the uterine cervix, being inversely linked with ERα status. Detection of aberrant pS2 expression may be useful for the early diagnosis of C-ACas, including AIS lesions.

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