Oestrogen receptor β expression and depth of myometrial invasion in human endometrial cancer

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Summary We assessed the relative expression of oestrogen receptor (ER)α and oestrogen receptor (ER)β mRNAs in 36 human endometrial cancers using a multiplex polymerase chain reaction (PCR). To determine whether or not the expression of ER subtypes in endometrial cancers is associated with clinicopathological parameters, we examined correlations between ER subtypes and age, tumour grade and depth of myometrial invasion. Using multiple regression analysis, myometrial invasion showed a significant correlation with ER-β: ER-α ratio (r = 0.54, *P* = 0.0007). The ER-β:ER-α ratio was high in advanced invasive carcinoma. Western blotting analysis showed that ER-β proteins were highly expressed in comparison with ER-α proteins in endometrial cancer with severe myometrial invasion. Our results suggest that ER-β is important in the progression of myometrial invasion. © 2001 Cancer Research Campaign   htt://www.bjcancer.com

Keywords: endometrial cancer; oestrogen receptor alpha and beta; myometrial invasion

Oestrogen receptors (ER) are encoded by the ER-α and ER-β genes (Kuiper et al, 1996; Mosselman et al, 1996). These two receptors share about 95% homology in the DNA binding domain and 55% homology in the ligand binding domain, bind to a consensus oestrogen response element (ERE) (Tremblay et al, 1997) and have similar ligand binding properties (Kuiper et al, 1997). The reverse transcription polymerase chain reaction (RT-PCR) has revealed that ER-β is expressed at high levels in the prostate and ovary (Kuiper et al, 1996; Mosselman et al, 1996), and at moderate levels in many other tissues, including the testis and the uterus, parts of which also seem to express ER-β (Kuiper et al, 1997). Oestrogen promotes the progression of breast cancer. The presence of ER-α and ER-β mRNA in both normal and neoplastic human breast tissues has been reported (Dotzlaw et al, 1997). Furthermore, the relative expression of ER-α and ER-β mRNA differs between normal human breast tissues and concurrently matched ER-positive breast tumours (Leygue et al, 1998), suggesting that ER-α and ER-β expression is functionally altered during breast tumorigenesis. Although the two receptors are often coexpressed in breast cancer, the levels of ER-β mRNA appear to vary among breast tumours (Dotzlaw et al, 1997), raising the question as to whether ER-β expression correlates with prognostic or endocrinological markers. The expression of ER-β mRNA is inversely correlated with progesterone receptor (PR) status, suggesting that ER-β expression is regulated by progestins (Dotzlaw et al, 1999). However, no specific data regarding ER-β distribution in endometrial cancer have been published. Therefore, we assessed the relative expression of ER-α and ER-β mRNAs in endometrial cancers, to determine whether or not the expression of these receptors is altered during endometrial tumorigenesis.

MATERIAL AND METHODS

Tissues and RNA extraction

We examined samples of 35 endometrial cancers, 1 of complex hyperplasia and 1 normal endometrium in the secretory phase obtained with consent from patients who were treated at the Gunma University Hospital, Department of Obstetrics and Gynaecology. Samples were immediately frozen in liquid nitrogen and stored at –80°C. No chemotherapy or radiation therapies were performed prior to tumour excision. Surgically resected tissues were sampled for histopathological diagnosis. Clinical stage and histological classification were established according to the International Federation of Gynaecology and Obstetrics (FIGO) 1988 criteria. Myometrial invasion was classified into 7 grades. Grade 0 indicates a lack of myometrial invasion. Depth of myometrial invasion was measured microscopically and categorized from grades 1/6 to 6/6, 6/6 means carcinoma reached the serosa. Details are presented in Tables 1 and 2. Total RNA was extracted from frozen tissue sections using the GLASS MAX RNA Micro-isolation Spin Cartridge System (Gibco/BRL, Grand Island, NY) according to the manufacturer's instructions.

Primers and RT-PCR conditions

Total RNA (1 μg) was reverse transcribed in 20 μl of reaction mix for 59 min at 42°C, then for 15 min at 70°C. The reaction mix contained 50 units of Moloney murine leukaemia virus reverse transcriptase (Perkin Elmer, Foster City, CA), 0.8 mM dNTPs (Perkin Elmer), 20 units of RNase inhibitor (Perkin Elmer), 10 mM Tris-HCl (pH8.3), 50 mM KCl, 1.5 mM $MgCl_2$ and 2.5 μM random hexamers (Perkin Elmer). To determine the relative expression of ERα and ERβ mRNA within individual samples, two sets of primers were added to each reaction and both ERα and ERβ cDNAs were co-amplified. Reverse transcription reaction (3 μg) was amplified by PCR in a final volume of 25 μl, containing 1 unit of Ampli Taq Gold (Perkin Elmer), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 2 μM of

Table 1 Pathologic details of endometrial cancers used in this study

Clinical parameters		No.
Patients	Total no. in study	36
	Mean age (range)	56 (36~81)
	<50 years	10
	≧50 years	26
Tumour histology	endometrioid	32
	clear cell	1
	adenosquamous	1
	serous	1
	complex hyperplasia	1
Tumour grade	Grade 1	19
	Grade 2	9
	Grade 3	4
	Others	4
Myometrial invasion	0	1
	1/6	19
	2/6	7
	3/6	3
	4/6	2
	5/6	1
	6/6	3
Clinical stage	Ia	0
	b	22
	c	3
	IIa	4
	b	2
	IIIa	2
	b	0
	c	1
	IV	1

each primer under the following conditions: 95°C for 10 min followed by 30 cycles at 94°C for 30 sec and 72°C for 3 min and an extension step at 72°C 5 min. Under these conditions, PCR products were generated within a linear range. Primers used to amplify ER-α were: (sense) 5′-CTGGCTACATCATCTCGGTTCCGCA-3′ (nucleotides 1577~1601); (antisense) 5′-TCAGGCCTGCCTTG-GCCATCAGGT-3′ (nucleotides 1755~1778), generating an amplified product of 202 bp. For ER-β, primer sequences were: (sense) 5′- GCGCTCAATTGACCACCCCGGCAA-3′ (nucleotide 894~927); (antisense) 5′-GCATCGGTCACGGCGTTCAGCAAG-3′ (nucleotide 1143~1166), generating an amplified product of 273 bp. The ubiquitously expressed GAPDH cDNA was amplified in parallel, to prove that used RNAs were not degraded.

PCR products were subcloned into the pGEM-T Easy vector (Promega, Madison, WI) and sequenced using a cycle sequencing kit (Takara Shuzo; Shiga, Japan).

Multiplex PCR validation

Two tumours (T1 and T2) were used to validate a multiplex RT-PCR that was designed to determine the relative expression of ER-β: ER-α. T1 expressed medium ER-β/high ER-α mRNA levels, on the other hand, T2 expressed low ER-β/high ER-α mRNA levels. 6 cDNA preparations were prepared containing varying percentages of T1 and T2 cDNA by mixing 5, 4, 3, 2, 1 and 0 μl of T1 cDNA with 0, 1, 2, 3, 4 and 5 μl of T2 cDNA (100, 80, 60, 40, 20 and 0% T1 cDNA, respectively). The two receptor subtypes were coamplified in 3 μl of cDNA preparation as described above. PCR products were separated on 1.8% Metaphor agarose gels (FMC Bio Products; Rockland, USA) and visualized by ethidium bromide staining under UV illumination.

Quantitation and statistical analysis

To compare ER-β mRNA expression relative to that of ER-α mRNAs, ER-α and ER-β cDNA were coamplified as described above. Images were captured under UV transillumination on type 667 film (Polaroid Co, Cambridge, MA) and signals were quantified using BAS-2000 imaging analyser (Fuji Photo Film Co, Tokyo Japan). Statistical analysis was performed using the Stat Flex for Windows (Artech Inc, Osaka, Japan). Multiple regression method was used to analyse the association between the β:α ratio and clinical factors including age, tumour grade, clinical stage and myometrial invasion.

Western blot analysis

Of 37 endometrial tissue samples, 34 were analysed by Western blotting. The samples were homogenized in $1 \times$ SDS buffer (2% SDS, 100 mM DTT and 60 mM Tris-HCl pH 6.8). Then, the preparations were boiled for 5 min, and passed through a 26G needle with a 1 ml syringe. After monitoring at OD 280/260, they were diluted with $1 \times$ SDS buffer, and 0.4 units (OD280 = 1 unit) of each sample was applied to SDS-PAGE. Gels were soaked in transfer buffer (48 mM Tris-HCl, 30 mM glycine and 20% methanol, pH 9.2) and proteins were transferred to nitrocellulose membranes (Hybond ECL, Buckinghamshire, England). Immunoblotting was performed using anti-ER-α, anti-ER- β, anti-PR (Santa Cruz Biotechnology; Santa Cruz, CA) and anti-β-actin (SIGMA; Japan). Signals were developed using ECL Western blotting detection reagents (Amersham Pharmacia Biotech UK Limited).

RESULTS

Multiplex PCR validation

The relative expression of ER-α and ER-β mRNA within individual samples was determined using a multiplex PCR assay. Two sets of primers were added to each PCR mixture and both ER-α and ER-β were amplified in a single tube. To determine whether or not the results obtained from this assay accurately reflected the initial ER-β:ER-α ratio, a preliminary multiplex PCR was performed on tumours T1 and T2. Tumour T1 expressed medium ER-β/high ER-α mRNA levels, whereas T2 expressed low ER-β/ high ER-α mRNA levels. As shown in Figure 1A, the PCR signal corresponding to ER-β decreased with decreasing input of T2 cDNA, and the ER-α signal increased with increasing input of T1 cDNA. The ER-β:ER-α ratio signals were plotted as a function of the percentage of T1 cDNA input. The ER-β:ER-α ratio increased in a linear fashion as the ER-β input increased. Multiplex PCR under these conditions showed a dose related increase until the relative expression of ER-β and ER-α was <0.5 (Figure 1B).

Expression of ER-α and ER-β mRNAs in human endometrial cancer tissues, and association with clinical information

35 endometrial cancer samples, 1 of complex hyperplasia and 1 normal endometrium were analysed and the expression of both ER-α and ER-β was compared by multiplex PCR (Figure 2). The 202 bp and 273 bp DNA fragments from a tumour sample that emitted intense ER-α and ER-β signals were subcloned and

Table 2 Clinical characteristics of patients in this study

Case	Age	Histology	Clinical stage	Histological grade	Myometrial invasion	Surgical procedure	Adjuvant therapy	Outcome	β/α ratio ×100
1	58	endometrioid	Ib	G1	2/6	mRH,BSO,PLA	CAP2	death(3mo)*¹	7.7
2	50	endometioid	Ib	G1	1/6	mRH,BSO,PLA		dfs(2y7mo)	0.0
3	81	endometrioid	IIa	G3	2/6	ATH,BSO		death(2y5mo)	2.5
4	48	endometrioid	IIa	G2	1/6	ARH,BSO,PLA		dfs(2y9mo)	7.6
5	58	endometrioid	Ib	G3	1/6	mRH,BSO,PLA	CAP3	dfs(2y5mo)	11.0
6	80	clear cell	Ic		5/6	ATH,BSO		dfs(2y8mo)	47
7	44	adenosquamous	Ib		1/6	mRH,BSO,PLA,PAN		dfs(2y6mo)	6.8
8	60	endometrioid	Ib	G3	1/6	ATH,BSO	CAP3	dfs(2y8mo)	0.0
9	51	endometrioid	Ib	G2	1/6	ATH,BSO	CAP5	dfs(2y4mo)	6.3
10	55	endometrioid	Ib	G1	3/6	mRH,BSO,PLA		dfs(2y2mo)	31.5
11	47	endometrioid	Ib	G1	1/6	mRH,BSO,PLA		dfs(2y1mo)	1.5
12	74	serous adeno	IIIc		1/6	mRH,BSO,PLA	radiation	death(1y6mo)	6.0
13	52	endometrioid	IIb	G1	2/6	mRH,BSO,PLA		dfs(2y0mo)	5.6
14	45	endometrioid	IIa	G1	2/6	mRH,BSO,PLA	CAP3	dfs(1y8mo)	12.2
15	36	endometrioid	Ib	G1	2/6	ATH,BSO		death(POD6)*²	4.3
16	46	endometrioid	Ib	G1	1/6	mRH,BSO,PLA	CAP3	dfs(1y7mo)	8.2
17	69	endometrioid	IIa	G2	6/6	mRH,BSO,PLA	CAP3	dfs(1y7mo)	53.4
18	51	endometrioid	Ib	G1	1/6	mRH,BSO,PLA	CAP5	dfs(1y5mo)	14
19	50	endometrioid	Ib	G2	3/6	mRH,BSO,PLA	CAP	dfs(1y6mo)	19
20	50	endometrioid	Ib	G1	1/6	mRH,BSO,PLA	CAP3	dfs(1y7mo)	3.4
21	54	endometrioid	Ib	G1	1/6	mRH,BSO,PLA		dfs(2y5mo)	0.0
22	50	endometrioid	Ib	G1	1/G	mRH,BSO,PLA	CAP4	dfs(1y10mo)	0.0
23	74	endometrioid	IIIa	G1	6/6	ATH,BSO		dfs(2y6mo)	16
24	53	endometrioid	Ib	G1	1/6	mRH,BSO,PLA		dfs(1y6mo)	1.5
25	47	endometrioid	Ib	G2	2/6	mRH,BSO,PLA	CAP3	dfs(1y6mo)	5.4
26	60	endometrioid	Ib	G2	1/6	ARH,PLA	CAP3	dfs(1y5mo)	11.1
27	58	endometrioid	Ib	G1	1/6	mRH,BSO,PLA		dfs(1y5mo)	9
28	65	endometrioid	Ib	G1	1/6	mRH,BSO,PLA		dfs(1y4mo)	16.4
29	70	endometrioid	IV	G3	6/6	probe lapa	CAP,TJ	death(2y5mo)	31
30	53	endometrioid	Ib	G2	4/6	mRH,BSO,PLA	CAP	dfs(1y4mo)	16.8
31	61	endometrioid	Ib	G1	2/6	mRH,BSO,PLA		dfs(2y8mo)	16.8
32	61	endometrioid	Ic	G1	4/6	ATH,BSO		unknown	29
33	41	complex hyperplasia			p 0	ATH,BSO		dfs(3y8mo)	1.2
34	49	endometrioid	IIIa	G2	3/6	mRH,BSO,PLA	CAP	dfs(2y6mo)	4.5
35	41	endometrioid	IIb	G1	2/6	ARH,BSO,PLA	radiation	dfs(1y3mo)	5
36	70	endometrioid	Ic	G2	1/6	mRH,BSO,PLA		dfs(1y3mo)	1.5
37	50	normal(secretary phase)				ATH			14.6

endometrioid, endometrioid adenocarcinoma; adenosquamous, adenosquamous carcinoma; serous adeno, serous adenocarcinoma; clear cell, clear cell adenocarcinoma; mRH, modified radical hysterectomy; BSO, bilateral salpingo-oophorectomy; PLA, pelvic lymphadenectomy; PAN, para-aortic lymphadenectomy; ARH, abdominal radical hysterectomy; CAP, cyclofosphamide-farmorubicin-cisplatin; TJ, paclitaxel-carboplatin; dfs, desease free survival; pos, postoperative survival; *¹Patient died of cerebral infarction, *²Patient died of pulmonary embolism.

sequenced. The tumour sequence was identical to that published for human ER-α and ER-β (Mosselman et al, 1996). ER-α was expressed constantly in all samples, whereas ER-β was expressed to varying degrees (Figure 2B). An ER-β:ER-α ratio of >0.5 was found in only one sample in the present study (Figure 2A). To determine whether or not ER subtype coexpression in endometrial cancers is associated with clinical parameters, we examined multiple regression analysis. Only myometrial invasion showed a significant correlation with the ER-β:ER-α ratio ($r = 0.54$, $P = 0.0007$) (Table 3). Figure 3 shows the correlation between the ER-β:ER-α ratio and depth of myometrial invasion. Spearman rank correlation test determined that the correlate coefficient was 0.786 ($P < 0.0001$). In one normal endometrium, the ER-β:ER-α ratio was in the medium range (0.145).

Western blotting analysis

We examined the status of ER-α and ER-β protein by Western blotting in the 34 endometrial cancer tissue samples. The representative pattern of Western blotting was shown in Figure 2C. The relative expression of ER-β and ER-α protein was well correlated with analysis of mRNA. Among the 7 cases with severe myometrial invasion (depth ≥ 3/6), which expressed high ER-β:ER-β mRNA ratio (β/α ratio ≥ 0.16), 6 cases expressed ER-β protein relatively more strongly than ER-β protein (No. 6, 10, 17, 19, 23 and 30). Additionally, we analysed progesterone receptor (PR) protein expression, as some investigators have reported that expression of the progesterone receptor is related to good prognosis. PR protein was expressed in all endometrial cancer tissues to varying degrees, and was not correlated with depth of myometrial invasion (Figure 2C). There appeared to be no correlation between the PR protein expression pattern and clinicopathological parameters.

DISCUSSION

Oestrogens are important mitogenic stimulants in endometrial and breast cancer in addition to playing important physiological roles in normal tissues. Oestrogen acts by binding to its specific receptors, ER-α and ER-β, although the physiological roles of these receptors have not been clearly distinguished. Dotzlaw et al (1997) showed that ER-α expression does not correlate with that of ER-α,
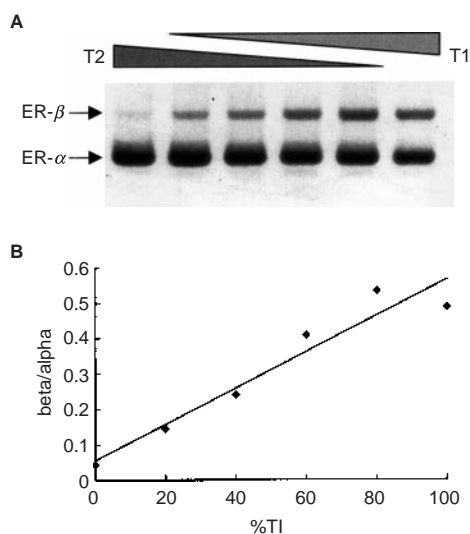
A

B

Figure 1 Multiplex amplification of T1 (moderate ER-β/high ER-α content) and T2 (low ER-β/high ER-α content) cDNA mixed preparations. (**A**) Increasing amounts of T1 cDNA and decreasing amounts of T2 cDNA were mixed and amplified by PCR using ER-α and ER-β specific primers in a single tube. (**B**) PCR products were separated on 1.8% metaphor agarose gels and stained with ethidium bromide

and that both ER-α positive (T-47D, T-47D-5) and negative (MDA MB231, MCF 10A1) cell lines express ER-β. They suggested that ER-β expression plays a possible role in human breast cancer. Leygue et al (1998) used multiplex-PCR and a ligand binding assay to show that the ER-α:ER-β ratio significantly increases in tumour components compared with normal components. Therefore, this ratio might play a role in the alteration of oestrogen action that occurs during the development of human breast cancer. In addition, Speirs et al (1999) showed that most breast tumours express ER-β, either alone or in combination with ER-α, and that those tumours coexpressing ER-α and ER-β were node positive and tended to be of higher grade. Thus, the clinical importance of measuring ER-β levels in breast cancer has been established. However, the relevance to endometrial cancer has not been determined.

We are the first to study ER-α and ER-β mRNA coexpression in human endometrial cancer tissue using multiplex RT-PCR and to demonstrate a correlation between the ER-β:ER-α ratio and clinicopathological parameters. We found that ER-α mRNA was expressed in all endometrial carcinomas examined. While the expression of ER-β mRNA varied among tumours and the level of ER-β mRNA was relatively high especially in advanced invasive carcinomas, the ER-β:ER-α mRNA ratio significantly correlated with the depth of myometrial invasion. Western blotting analysis also showed that ER-β proteins were highly expressed in comparison with ER-α proteins in endometrial cancer with severe myometrial invasion. The depth of myometrial invasion is a potent risk factor for endometrial cancer and positively correlates with prognosis (Le Vacchia et al, 1983; Morrow et al, 1991). Therefore, preoperative knowledge of the precise depth of myometrial invasion undoubtedly benefits surgical planning. Ultrasound and MRI imaging or measurement of serum CA125 levels are useful, but when endometriosis or leiomyoma are complicating factors, such findings are not sufficient. The results of the present study demonstrated that understanding the ER-β:ER-α ratio could be beneficial for predicting the depth of myometrial invasion.
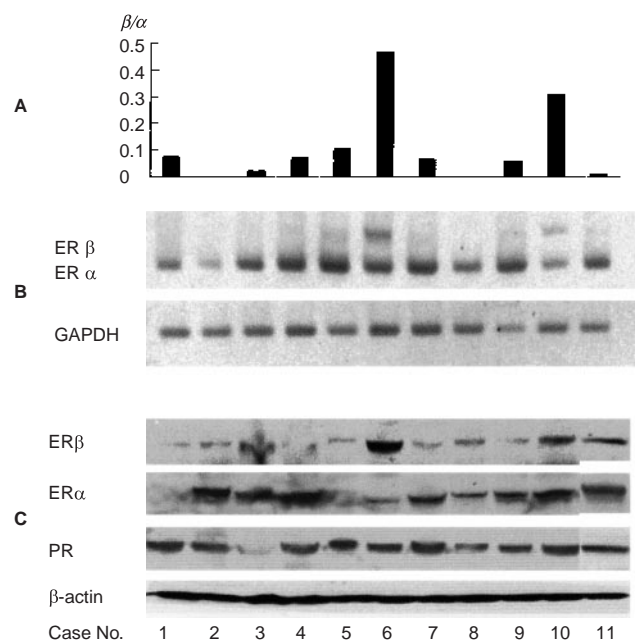
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Figure 2 (**A**) The ratio of ER-β/ER-α mRNA expression in multiplex RT-PCR. (**B**) Multiplex RT-PCR analysis of ER-α and ER-β expression. (**C**) Western blotting analysis of ER-α, ER-β and PR protein. The relative expression of ER-β and ER-α protein were almost correlated with analysis of mRNA. PR protein was expressed in all samples to varying degrees. The case No. is concordant with that of Table 2
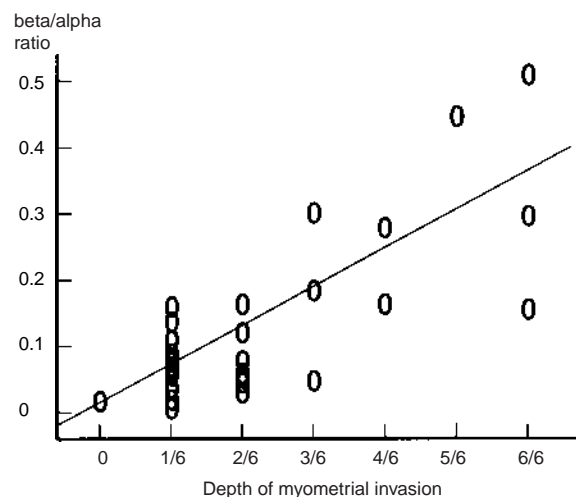

Figure 3 Analysis of the correlation between ER-β:ER-α ratio and depth of myometrial invasion. Depth of myometrial invasion was categorized into 7 groups, from 0: no invasion to 6/6: reached the serosa. The correlation coefficient was 0.786 ($P < 0.0001$). The association was assessed by Spearman's rank correlation coefficient. P values were the result of two-sided test ($rS = 0.634$ and $t = 4635$ (df = 32)).

The mechanisms behind how the expression of ER-β causes deep endometrial invasion are difficult to define. ER subtypes can signal not only from the classical oestrogen response element but also from an AP1 enhancer element (Gaub et al, 1990; Paech et al, 1997). Therefore, ER-β may elicit its own signals and interfere with those of ER-α. Theoretically, the growth of ER positive cells can be inhibited by antioestrogens, such as tamoxifen or the pure antioestrogen, ICI 182780. Tamoxifen is currently the first-line therapy for treatment of ER positive breast cancer (Osborne et al,

Table 3 Multiple regression analysis of factors related to ER-β:ER-α ratio

	Coefficient	Standard error	P value
Age	– 0.003188	0.0193	0.8706
Tumour grade	– 0.015132	0.2497	0.9521
Clinical stage	– 0.158564	0.1362	0.2548
Myometrial invasion	0.542333	0.1419	0.0007

$R^2 = 0.4178$.

1998). However, some patients who initially respond to tamoxifen eventually go into relapse, probably due to the acquisition of antioestrogen resistance. ER-β positivity tends to be associated with more poorly differentiated breast cancers (Speirs et al, 1999) and it has been shown that ER-β is overexpressed in tamoxifen resistant breast tumour (Speirs et al, 1999). Therefore, the coexpression of ER subtypes in the same tumour may modify the response to oestrogen and the tumour may become deeply invasive even in a low oestrogen environment. On the other hand, tamoxifen acts as an antioestrogenic and as an oestrogen-like substance on the endometrium (Hyder et al, 1996; Berliere et al, 1998), but its molecular mechanism has not yet been confirmed. The expression of ER-α and ER-β may decide the nature of the endometrial cell response to tamoxifen.

Many studies have suggested that the progesterone receptor (PR)/ER concentration is significant as a prognostic parameter for endometrial cancer (Friberg and Noren, 1993; Kadar et al, 1993; Morris et al, 1995; Moutsatsou and Sekeris 1997), and that PR status significantly predicts the disease-free survival of patients with endometrial cancer. It has been shown that lymph node metastasis from endometrial cancer is correlated with negative PR protein expression (Iwai et al, 1999). Therefore, the effect of ER-β on the expression of PR as well as the mechanism of how ER-β affects the nature of endometrial cancer should be clarified. Based on our result, PR protein expression appeared not to be associated with that of ER subtypes and clinicopathological parameters. Only one sample was node positive in our study. Thus, this issue needs to be investigated in studies involving more cases with nodal involvement.

We reported that ER-β/ER-α expression ratio was useful as a novel prognostic indicator. The patients in this study were followed for a limited duration after operation, and thus whether or not ER-β status actually indicates disease-free survival must be established.

In conclusion, although the biochemical function of ER-β in relation to endometrial cancer remains unknown, our results suggest that ER-β plays an important role in the progress of myometrial invasion. If so, ER-β may be an useful prognostic tool for patients with endometrial carcinoma.

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