

Figure 3. Demethylation analysis of *CHFR* in SNG-II cells. (A), In MSP, bands due to aberrant hypermethylation reduced with time after 5-aza-dC administration. (B), RT-PCR analysis showed recovered *CHFR* expression 24 h after the second 5-aza-dC administration. M, marker; PC, positive control; NC, negative control.

Table VI. Changes in sensitivity (T/C ratio) of endometrial cancer-derived cells to various anticancer drugs after treatment with a demethylating agent.

Cell line	<i>CHFR</i>	Cisplatin (%)		Doxorubicin (%)		Paclitaxel (%)		Docetaxel (%)	
		5-aza(-)	5-aza(+)	5-aza(-)	5-aza(+)	5-aza(-)	5-aza(+)	5-aza(-)	5-aza(+)
SNG-II	M	52.0	66.6	65.1	80.5	16.7	60.8	15.1	61.5
Hec108	M	56.6	64.6	76.6	76.7	11.8	70.7	20.6	69.4
KLE	U	57.6	64.5	93.6	100	60.8	75.2	63.9	72.9

5-aza, 5-aza-dC; M, methylated; U, unmethylated.

four G3 patients, and 75.0% (3/4) of these specimens showed reduced *CHFR* expression (Fig. 5). To investigate the relationship of sensitivity to paclitaxel with aberrant hypermethylation of *CHFR*, 12 surgical specimens of endometrial cancer were examined using CD-DST. The T/C ratio of specimens showing aberrant hypermethylation of *CHFR* was the lowest (37.6%), and these specimens exhibited higher sensitivity to paclitaxel compared to specimens without aberrant hypermethylation (Table VII).

Discussion

Aberrant hypermethylation of *CHFR* has been reported in gastrointestinal and lung cancers (7-9), but has not been examined in gynecologic cancer. Furthermore, the relationship between aberrant hypermethylation of *CHFR* and biological and disease characteristics has not been examined in any cancer. Our results indicate that aberrant hypermethylation of *CHFR* occurs in G3 adenocarcinoma significantly more frequently than in G1 adenocarcinoma ($p < 0.05$, 28.6%).

It is known that *CHFR* negatively regulates *Aurora-A* mitotic kinase: *Aurora-A* is induced by inhibition of expression of *CHFR* (10), and overexpression of *Aurora-A* has been shown to induce chromosomal instability (CI) in various cancers (11-13). Since CI in endometrial cancer is commonly found in G3 adenocarcinoma (65%) (14), it is likely that aberrant hypermethylation of *CHFR* is one cause of induction of CI in endometrial cancer. In addition, a relationship between aberrant hypermethylation of *CHFR* and *hMLH1* has been reported in colon cancer (15), and aberrant hypermethylation of *hMLH1* has also been found in approximately 40% of endometrial cancers and is thought to be involved in the early stage of carcinogenesis (16,17).

In cell cycle analysis using flow cytometry in endometrial cancer-derived cell lines, cells with a normal *CHFR* gene showed cell accumulation in G2/M phase after paclitaxel administration. In contrast, in cells with *CHFR* inactivated by aberrant hypermethylation, cell accumulation in G2/M phase was not observed after paclitaxel administration but was found after combined treatment with paclitaxel and a demethylating

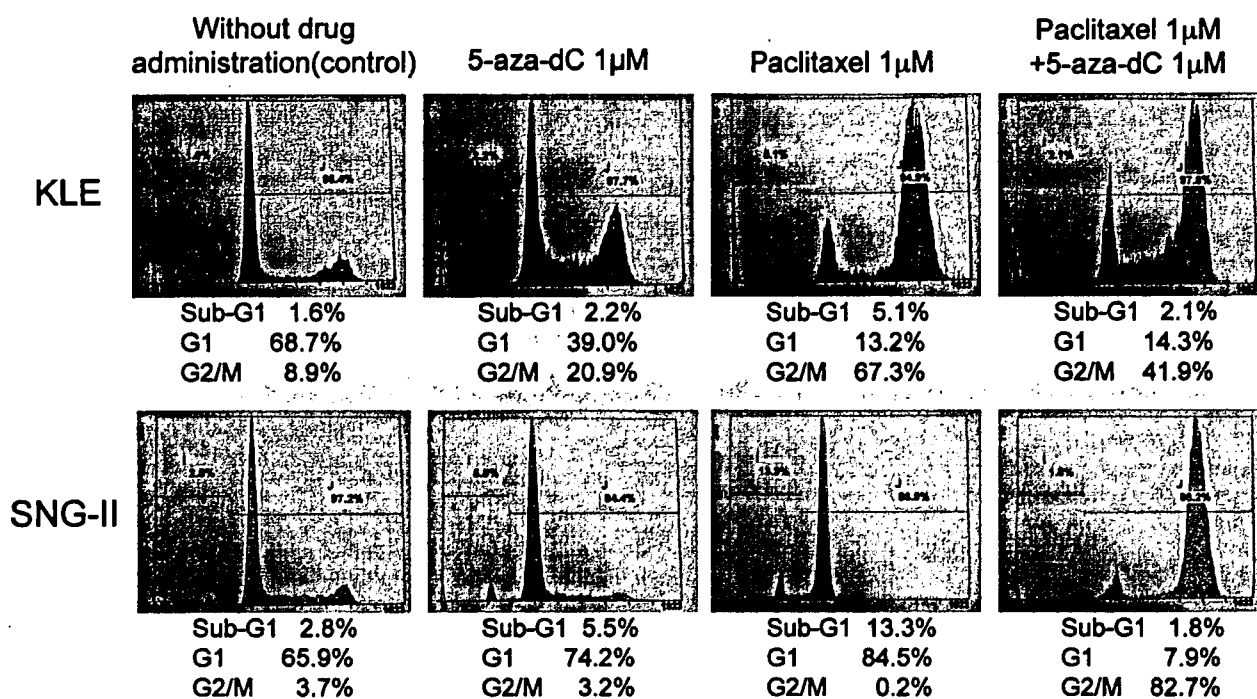


Figure 4. Cell cycle analysis in KLE and SNG-II cells using flow cytometry. KLE cells treated with paclitaxel alone and with combined paclitaxel and 5-aza-dC showed cell accumulation in G2/M phase and no significant change in the number of cells in Sub-G1 phase. SNG-II cells treated with paclitaxel alone exhibited no cell accumulation in G2/M phase, but an increased number of cells in Sub-G1 phase; however, SNG-II cells treated with combined paclitaxel and 5-aza-dC showed significant cell accumulation in G2/M phase and a low number of cells in Sub-G1 phase, similarly to the controls.

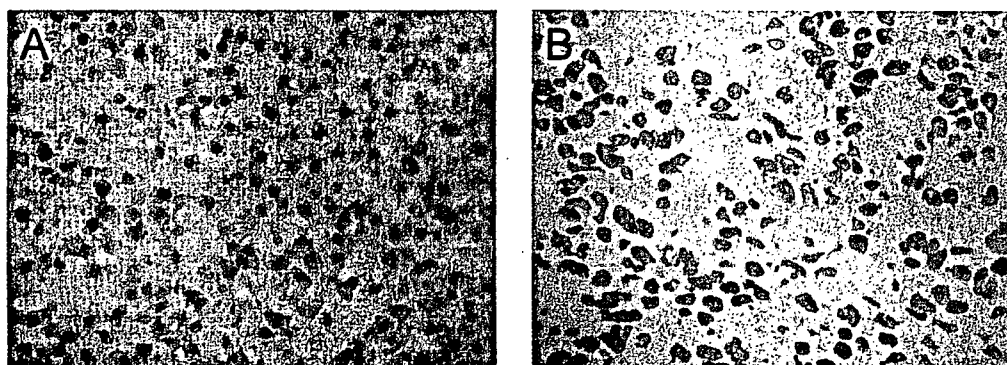


Figure 5. Immunohistochemical analysis of CHFR protein expression in patients with endometrioid adenocarcinoma (G3) with aberrant hypermethylation of *CHFR*. (A), EC35 staining; (B), EC32 staining. The nuclei of one of the four G3 adenocarcinoma patients stained positively (A), but three patients had nuclei with reduced staining (B).

agent. These results suggest that cells with normal *CHFR* undergo G2 arrest and can repair damage when the cells are treated with taxanes, thereby exhibiting resistance to taxanes, whereas cells with *CHFR* inactivated by aberrant hypermethylation cannot detect damage and enter mitosis, thereby showing high sensitivity to taxanes. Based on the increased number of cells in Sub-G1 phase without accumulation of G2/M cells after paclitaxel administration, the high taxane sensitivity of cells with inactivated *CHFR* appears to be due to mitotic catastrophe causing cell death after entry into the mitotic phase.

Cells with aberrant hypermethylation of *CHFR* showed greatly decreased sensitivity to paclitaxel in the CD-DST after demethylation treatment. Although 5-aza-dC adminis-

tration will result in demethylation of many genes, in addition to *CHFR*, sensitivity to cisplatin or doxorubicin was unaltered after demethylation, whereas sensitivity to paclitaxel and docetaxel increased, suggesting that aberrant hypermethylation of *CHFR* is of importance in sensitivity to taxanes. Thus, aberrant hypermethylation of *CHFR* is a potential index for prediction of cell sensitivity to taxanes.

The CD-DST is an anticancer drug sensitivity test in which tumor cells are cultured three-dimensionally. This method requires only a small number of cells and both culture cell lines and tumor tissues can be analyzed in the same experimental system. Use of the CD-DST for gynecologic cancer has been reported (6), and here we used the CD-DST in analysis of taxane sensitivity of clinical specimens of

Table VII. Aberrant hypermethylation of *CHFR* and sensitivity (T/C ratio) of specimens of endometrial cancer to paclitaxel.

No.	Tissue type	Stage	Differentiation	<i>CHFR</i>	T/C ratio (%)
EC30	AS	Ib	G1	U	84.0
EC31	EM	Ib	G2	U	79.6
EC32	EM	IIIc	G3	M	37.6
EC33	EM	Ib	G2	U	86.9
EC36	AS	IIIc	G3	U	59.8
EC37	EM	Ic	G3	U	38.2
EC38	AS	IIIc	G1	U	66.4
EC39	EM	Ic	G1	U	100
EC47	EM	IIIc	G3	U	100
EC48	EM	IIIc	G2	U	84.3
EC49	EM	Ib	G1	U	79.7
EC50	EM	IVb	G2	U	70.3

EC, endometrial cancer; EM, endometrioid adenocarcinoma; AS, adenosquamous carcinoma; G1, well-differentiated; G2, moderately differentiated; G3, poorly differentiated; M, methylated; U, unmethylated.

endometrial cancer. The CD-DST results for 12 specimens of endometrial cancer showed that cells with aberrant hypermethylation of *CHFR* had high sensitivity to paclitaxel, as also found in cultured cells; this is the first such study of this relationship in endometrial cancer. Immunohistochemical analysis showed that of four patients with G3 adenocarcinoma with aberrant hypermethylation of *CHFR*, three exhibited reduced expression of *CHFR* protein. This result indicates that aberrant hypermethylation of *CHFR* is involved in reduced protein expression *in vivo*. Overall, our results suggest a new strategy for design of personalized medicine for endometrial cancer using the hypermethylation status of *CHFR* as a molecular index. This strategy may be particularly important in treatment of G3 adenocarcinoma, since it is known to have a poor prognosis.

Acknowledgements

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A validation study of a scoring system to estimate the risk of lymph node metastasis for patients with endometrial cancer for tailoring the indication of lymphadenectomy[☆]

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Abstract

Objective. The aim of this study was to verify whether a preoperative scoring system to estimate the risk of lymph node metastasis (LNM) in endometrial carcinoma is clinically useful for tailoring the indication of lymphadenectomy.

Study design. LNM score was set up using volume index, serum CA125 level, and tumor grade/histology, which were found to be independent risk factors for LNM in a pilot study. Based on the LNM score before a validation study was started, the estimated rates of LNM (para-aortic LNM) were 3.4% (0.0%) in a low risk group, 7.7% (5.8%) in an intermediate group, 44.4% (30.6%) in a high risk group and 70.0% (50.0%) in an extremely high risk group. The validation study was carried out using data for 211 patients with endometrial carcinoma for whom three risk factors were preoperatively confirmed. Logistic regression analysis was used to determine whether these factors remain valid. The actual rate of LNM was investigated according to the LNM score.

Results. Volume index, serum CA125 level, and tumor grade/histology were found to be independent risk factors for LNM in the cohort of this study. The actual rates of LNM (para-aortic LNM) were 3.2% (1.0%) in the low risk group, 15.3% (11.9%) in the intermediate group, 30.2% (23.8%) in the high risk group and 78.6% (57.1%) in the extremely high risk group.

Conclusion. The actual rate of LNM for each score was fairly consistent with the estimated rate of LNM. Para-aortic lymphadenectomy may not be necessary in cases of a low risk group. A large prospective multicenter clinical trial needs to be conducted to establish the clinical usefulness of our preoperative scoring system.

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Keywords: Endometrial carcinoma; Lymph node metastasis; MRI; CA125

Introduction

Retroperitoneal lymph node metastasis (LNM) is a critical prognostic factor for patients with endometrial carcinoma [1]. Lymphadenectomy has become accepted as the standard

treatment for women with endometrial cancer but still has some issues of debate. Many gynecologists would agree that patients with endometrial carcinoma who have grade 1 tumor with no myometrial invasion do not need lymphadenectomy [2]. However, there has not been a gold-standard method for selecting patients who do not need lymphadenectomy in a preoperative setting. It has been suggested that para-aortic lymphadenectomy has a potential therapeutic role in node-positive endometrial cancer [3]. However, a consensus has not been reached regarding the issue of whether to extend the

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application field of lymphadenectomy to the para-aortic area. We previously reported that volume index, which is a substitute for tumor volume, preoperative serum CA125 level, and histologic grade 3 tumor or serous adenocarcinoma determined by preoperative endometrial biopsy were independent risk factors for LNM [4]. Using these indexes, we formulated a scoring system to estimate the risk of LNM in endometrial cancer. The aim of this study was to determine whether this scoring system is valid in a different cohort of patients with endometrial cancer. Indication for lymphadenectomy is also discussed in this report.

Materials and methods

Study design

We designed a series of clinical studies to establish a scoring system that can determine the risk of LNM in an individual patient with endometrial cancer. These studies comprise of as follows: 1) a pilot study to define the independent risk factor of LNM that will be incorporated in the scoring system, 2) a validation study in which the scoring system will be applied to a different cohort of patients, and 3) a large multi-institutional observational study to verify the generalizability and applicability of the scoring system. This is a report of the validation study.

A pilot study for formulating lymph node metastasis score (LNM score)

The lymph node metastasis score (LNM score) was formulated based upon the data of 214 patients with endometrial cancer who underwent extensive surgical staging including systematic pelvic and para-aortic lymphadenectomy during the period between January 1993 and March 2000, which was reported in a previous paper [4]. In a pilot study, all patients with endometrial cancer underwent systematic lymphadenectomy. The pelvic lymph node groups that were dissected included the common iliac, external iliac, internal iliac, obturator, medial deep inguinal, lateral deep inguinal, parametrial, and sacral node group in the pelvic area. Para-aortic lymph nodes that were inferior to the level of the inferior mesenteric artery and para-aortic lymph nodes that were superior to the inferior mesenteric artery up to the level of the renal vessels were dissected. The clinicopathologic characteristics of the patients are shown in Table 1. Among the 214 patients, 31 (14.5%) had LNM and 19 (8.9%) had para-aortic LNM.

The scoring system includes volume index, serum CA125 level, and tumor grade/histology. Volume index was defined as the product of the maximum longitudinal diameter along the uterine axis, the maximum anteroposterior diameter (thickness) in a sagittal section image, and the maximum horizontal diameter in a horizontal section image. The results of measurements were used to obtain receiver operating characteristic (ROC) curves for LNM. Although we separately showed the ROC curve for pelvic LNM and that for para-aortic LNM in a previous report, the revised ROC curve for LNM that includes both pelvic LNM and para-aortic LNM is shown in Fig. 1. When determined on the curve, cut-off value for LNM was 36. The serum CA125 level was determined using a RIA kit (Fujirebio Diagnostics, Malvern, PA). The patient population was divided into two groups by age. Although we separately showed the ROC curve for pelvic LNM and that for para-aortic LNM in a previous report, the revised ROC curves for LNM that includes both pelvic LNM and para-aortic LNM are shown in Fig. 1. Using these curves, two cut-off values (70 U/ml for patients aged less than 50 years and 28 U/ml for patients aged 50 years or over) divided patients into low and high CA125 groups for LNM. Preoperative endometrial biopsy specimens were evaluated for tumor grade and histologic variant (three grades according to the 1988 FIGO criteria). Tumor grade/histology, which put two factors of tumor grade and histologic type together, was used as an independent variable in the revised analysis. In a previous report, we separately showed the results of logistic regression analysis in which pelvic LNM was used as a dependent variable and that in which para-aortic LNM was used as a

Table 1

Characteristics of a cohort of 214 patients with endometrial carcinoma to produce LNM score in the pilot study

Study design	Retrospective cohort study			
Number of institution	3			
Number of patients	214			
Age, median (range)	56 (23–80)			
Number of resected lymph nodes, mean±SD	71.2±34.0			
	No.	LNM	PLNM	PANM
pT (TNM classification)				
1a	54	0	0	0
1b	62	5	5	1
1c	44	8	8	6
2a	10	3	2	1
2b	8	5	5	4
3	31	5	4	4
4	5	5	5	3
Histologic type (preoperative diagnosis)				
Endometrioid	207	27	25	17
(G1)	(134)	(10)	(9)	(7)
(G2)	(55)	(10)	(10)	(4)
(G3)	(18)	(7)	(6)	(6)
Serous	7	4	4	2

These data were not published in a previous paper [4].

LNM: lymph node metastasis, PLNM: pelvic lymph node metastasis, PANM: para-aortic lymph node metastasis.

dependent variable. Table 2 shows the results of revised logistic regression analysis in which LNM including both pelvic LNM and para-aortic LNM was used as a dependent variable.

The impact of the LNM score was determined according to the number of independent risk factor which was confirmed in Table 2. All patients were classified into low risk group (with no risk factor), intermediate risk group (with one risk factor), high risk group (with two risk factors) and extremely high risk group (with all risk factors). Of the 214 patients, 116 (54%) were included in the low risk group, 52 (24%) were included in the intermediate risk group, 36 (17%) were included in the high risk group and 10 (5%) were included in the extremely high risk group. Based on the LNM score, the estimated rates of LNM (para-aortic LNM) were 3.4% (0.0%) in the low risk group, 7.7% (5.8%) in the intermediate group, 44.4% (30.6%) in the high risk group and 70.0% (50.0%) in the extremely high risk group.

Validation study of LNM score

Among patients with endometrial carcinoma treated in the Department of Obstetrics and Gynecology, Hokkaido University Hospital and twelve affiliated hospitals during the period from July 2000 to April 2005, 216 patients who underwent extensive surgical staging including systematic lymphadenectomy were entered in this study. The patients underwent pelvic MRI, endometrial biopsy, and serum CA125 level determination as preoperative examinations. Although lymphadenectomy was not carried out in some affiliated hospitals for some patients who were preoperatively diagnosed as having grade 1 tumor without myometrial invasion, all patients entered in the validation study underwent hysterectomy, bilateral salpingo-oophorectomy, and systematic lymphadenectomy as initial treatment. For a medical complication, para-aortic lymphadenectomy was not performed in one patient. Five cases with an admixture of sarcomatous component and carcinomatous component in a preoperative endometrial biopsy were excluded from this study. A total of 211 patients were included in the study. The clinicopathologic characteristics of those patients are shown in Table 3. The ages of the patients ranged from 24 to 77 years (median age, 57 years). 21 patients were in pT1a (TNM classification), 91 were in pT1b, 50 were in pT1c, 4 were in pT2a, 17 were in pT2b, 26 were in pT3, and 2 were in pT4. The lymphadenectomy specimens included a median of 77 lymph nodes (mean±SD, 79.4±29.6) evaluated for each patient. 36 (17.1%)

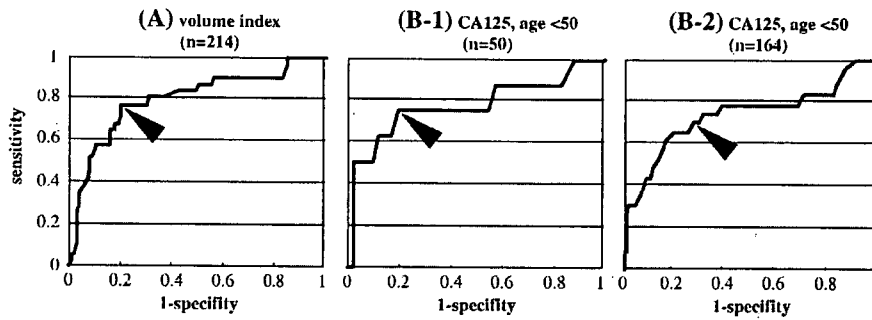


Fig. 1. (A) Receiver operating characteristic curve obtained from the relationships between volume index and LNM in the pilot study. Cut-off value (arrow) for LNM was 36. (B) Receiver operating characteristic curves obtained from the relationships between serum CA125 level and LNM. Cut-off value (arrow) for LNM was 70 U/ml for patients aged less than 50 years. Cut-off value (arrow) for LNM was 28 U/ml for patients aged 50 years or over. (These data were not published in a previous paper [4].)

patients had LNM and 26 (12.3%) had para-aortic LNM. Logistic regression analysis was used to determine whether the factors incorporated into the model of LNM score are still valid in a validation study. Volume index, MRI-based myometrial invasion, serum CA125 level, and tumor grade/histology were used as independent variables, and LNM was used as a dependent variable. The 211 patients were classified into a low risk group, an intermediate risk group, a high risk group and an extremely high risk group. The actual rates of LNM were compared to the estimated rates of LNM according to the model of LNM score.

Statistical analysis

Logistic regression analysis was used to select the risk factors for LNM. Variables that achieved statistical significance in univariate analysis were subsequently included in a multivariate analysis. The statistical significance level was set at .05. Statistical analyses were performed with StatView J-5.0 PPC (SAS Institute, Cary, NC).

Results

Cases with invasion of less than one half the myometrium had a sensitivity of 83%, a specificity of 83%, and an accuracy of 83%. Cases with the diagnosis of G1/G2 histopathology had a sensitivity of 97%, specificity of 74% and accuracy of 92%. Only 5.7% of cases with G1/G2 histopathology diagnosed by endometrial biopsy were upgraded to G3/serous adenocarcinoma after hysterectomy.

Table 4 shows the results of logistic regression analysis. Univariate analysis revealed that all factors were significantly related to LNM. Multivariate analysis confirmed that only high volume index, high serum CA125, and G3/serous adenocarcinoma were independent risk factors for LNM for this cohort of patients. MRI-based myometrial invasion had an odds ratio of 5.4 (95% confidence interval [CI]=2.4–11.9) before adjusting other factors but an odds ratio of 2.7 (95% CI=1.1–6.5) after adjusting volume index. Eventually it had an odds ratio of 2.0 (95% CI=0.8–5.3) after adjusting volume index, serum CA125, and tumor grade/histology.

LNM frequencies according to the LNM score are shown in Table 5. The rates of LNM were 3.2% (3/95) in the low risk group, 15.3% (9/59) in the intermediate group, 30.2% (13/43) in the high risk group and 78.6% (11/14) in the extremely high risk group. The rates of para-aortic LNM were 1.0% (1/95) in the low risk group, 11.9% (7/59) in the intermediate group, 23.8% (10/42) in the high risk group and 57.1% (8/14) in the extremely high risk group.

7 (6.2%) of the 113 patients who had G1/G2 tumor as assessed by endometrial biopsy and invasion of less than half of the myometrium as assessed by MRI had LNM. On the other hand, 3 (3.2%) of the 95 patients with low risk according to the LNM score had LNM. 5 (4.4%) of the 113 patients who had G1/

Table 2
Correlation between the factors incorporated into the LNM score in the pilot study

Factor	LNM		Univariate analysis <i>p</i> -value	Multivariate analysis			
	<i>n</i> / <i>N</i>	%		β	SE	OR (95% CI)	<i>p</i> -value
Preoperative tumor grade/histology							
G1/G2	20/189	10.6					
G3/serous	11/25	44.0	<0.0001	1.2	0.55	3.5 (1.2–10.2)	<0.05
Volume index							
<36	7/153	4.6					
≥36	24/61	39.3	<0.0001	1.8	0.53	5.7 (2.0–16.1)	<0.001
MRI myo-invasion							
<1/2	7/118	5.9					
≥1/2	24/96	25.0	<0.0005	0.7	0.53	2.1 (0.7–5.8)	NS
Serum CA125 level							
Low	9/146	6.2					
High	22/68	32.4	<0.0001	1.2	0.49	3.5 (1.3–9.1)	<0.05

These data were not published in a previous paper [4].

LNM: lymph node metastasis, PLNM: pelvic lymph node metastasis, PANM: para-aortic lymph node metastasis.

Table 3
Characteristics of a cohort of 211 patients with endometrial carcinoma included in the validation study

Study design	Retrospective cohort study			
Number of institution	13			
Number of patients	211			
Age, median (range)	57 (24–77)			
Number of resected lymph nodes, mean±SD	79.4±29.6			
	No.	LNM	PLNM	PANM
pT (TNM classification)				
1a	21	0	0	0
1b	91	11	10	10
1c	50	15	12	7
2a	4	1	1	1
2b	17	4	4	3
3	26	3	3	3
4	2	2	2	2
Histologic type (preoperative diagnosis)				
Endometrioid	205	33	30	24
(G1)	(124)	(13)	(12)	(8)
(G2)	(51)	(7)	(6)	(5)
(G3)	(30)	(13)	(12)	(11)
Serous	6	3	2	2

LNM: lymph node metastasis, PLNM: pelvic lymph node metastasis, PANM: para-aortic lymph node metastasis.

G2 tumor as assessed by endometrial biopsy and invasion of less than half of the myometrium as assessed by MRI had para-aortic LNM. On the other hand, only 1 (1.0%) of the 95 patients with low risk according to LNM score had para-aortic LNM.

Fig. 2 shows LNM frequencies for the combined cohorts of 425 patients with endometrial carcinoma according to LNM score. The rates of LNM were 3.3% (95% CI=0.9–5.7) in the low risk group, 11.7% (95% CI=5.7–17.7) in the intermediate group, 36.7% (95% CI=26.1–47.3) in the high risk group and 75.0% (95% CI=57.7–92.3) in the extremely high risk group. The rates of para-aortic LNM were 0.5% (95% CI=0.0–1.4) in the low risk group, 9.0% (95% CI=3.7–14.3) in the intermediate group, 27.6% (95% CI=17.6–37.7) in the high risk group and 54.2% (95% CI=34.2–74.1) in the extremely high risk group. Of the 211 patients in low risk group, 1.7%

Table 5
LNM frequencies according to LNM score

LNM score	RF	LNM (%)		PLNM (%)		PANM (%)	
		Pilot study	Validation study	Pilot study	Validation study	Pilot study	Validation study
Low risk	RF=0	3.4	3.2	3.4	3.2	0.0	1.0
Intermediate risk	RF=1	7.7	15.3	7.7	15.3	5.8	11.9
High risk	RF=2	44.4	30.2	38.9	23.3	30.6	23.8
Extremely high risk	RF=3	70.0	78.6	70.0	71.4	50.0	71.4

LNM: lymph node metastasis, PLNM: pelvic lymph node metastasis, PANM: para-aortic lymph node metastasis, RE: risk factor (MM tumor volume, serum CA125 level, tumor grade/histology).

(95% CI=0.0–4.1) with grade 1 carcinoma and invasion of less than half of the myometrium as assessed by MRI had LNM. On the other hand, 5.2% (95% CI=0.8–9.7) with grade 2 carcinoma or invasion of more than half of the myometrium as assessed by MRI had LNM.

Discussion

(G1) The FIGO 2001 annual report showed that the 5-year overall survival rate of patients with carcinoma of the endometrium has increased by 13.5% in the past 30 years [5]. The main change in the therapeutic paradigm for endometrial cancer in the past 30 years is the introduction of surgical staging. About 91% of patients have been surgically staged [5]. The treatment strategy including surgical staging may have resulted in improvement of the 5-year survival rate. Recent scientific publications have confirmed the relative safety of surgical staging including lymphadenectomy when performed by subspecialty trained surgeons, and this procedure has become accepted as the standard treatment for women with endometrial cancer [6]. However, there are still some issues of debate about lymphadenectomy in endometrial cancer. There has not been a gold-standard method for selecting patients who do not need lymphadenectomy in a preoperative setting, although many gynecological oncologists would agree that patients with endometrial carcinoma who have grade 1 tumor with no myometrial invasion do not need lymphadenectomy. A consensus

Table 4
Correlation between the factors incorporated into the LNM score and LNM in the validation study

Factor	LNM		Univariate analysis	Multivariate analysis			<i>p</i> -value
	<i>n/N</i>	%	<i>p</i> -value	β	SE	OR (95% CI)	
Preoperative tumor grade/histology							
G1/G2	20/175	11.4	<0.0001	1.6	0.47	4.8 (1.9–12.0)	<0.001
G3/serous	16/36	44.4					
Volume index							
<36	9/137	6.6	<0.0001	1.2	0.51	3.4 (1.3–9.4)	<0.05
≥36	27/74	36.5					
MRI myo-invasion							
<1/2	10/128	7.8	<0.0001	0.7	0.49	2.0 (0.8–5.3)	NS
≥1/2	26/83	31.3					
Serum CA125 level							
Low	11/134	8.2	<0.0001	1.0	0.47	2.7 (1.1–6.9)	<0.05
High	25/77	32.5					

LNM: lymph node metastasis, PLNM: pelvic lymph node metastasis, PANM: para-aortic lymph node metastasis.

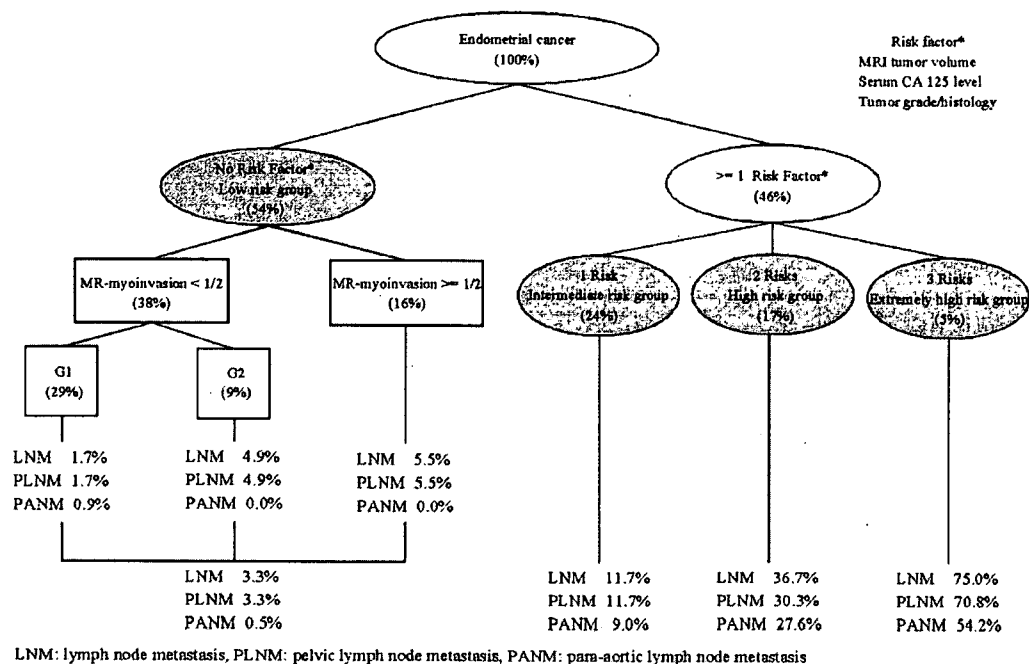


Fig. 2. Rate of LNM for the combined cohorts of 425 patients with endometrial carcinoma according to LNM score and distribution map of patients estimated from a previous paper [4].

has not been reached regarding the issue of whether to extend the application field of lymphadenectomy to the para-aortic area, although it has been suggested para-aortic lymphadenectomy has a potential therapeutic role in node-positive endometrial cancer. The establishment of a gold-standard method for selecting patients with endometrial carcinoma who have little risk for para-aortic LNM would be clinically very useful.

Classically, the risk of LNM has been classified according to the depth of myometrial invasion and histological grade. The methods used to evaluate those parameters in a preoperative setting are MRI and endometrial biopsy. Patients without myometrial invasion who have histologic grade 1 would be candidates for elimination of lymphadenectomy. Although myometrial invasion is indirectly evaluated by MRI in many institutions in Japan, the level of accuracy still has limitations. MRI-based evaluation used for diagnosis of deep invasion in a multi-institutional cooperative study had a sensitivity of 54% and specificity of 89%, indicating that results of previous single institutional studies might have been biased [7]. The level of accuracy for diagnosis of the presence or absence of myometrial invasion using MRI is poorer than the level of accuracy for diagnosis of deep (> 1/2) invasion. Pathological examination using frozen sections obtained during the operation improves the level of accuracy [8,9], but even its level of accuracy is not sufficient. It has been reported that only 60%–72% of patients who were diagnosed as having no myometrial invasion by pathological examination using frozen sections obtained during the operation were diagnosed as having no myometrial invasion by examination of resected specimens [8,9]. There is also the question of whether myometrial invasion is an independent risk factor of LNM. Since it is difficult to evaluate tumor volume in the resected uterus, tumor volume has not been included in

previous histopathologic analyses to determine independent risk factors of LNM. However, tumor volume can be estimated by using MRI. There has not been sufficient study to determine which is a more significant risk factor of LNM, myo-invasion or tumor volume. We showed in this validation study that myometrial invasion assessed by MRI might be a confounding factor of the volume index but not an independent risk factor for LNM, as we found in our previous study [4]. Although histological grade is evaluated by endometrial curettage in many institutions, the level of accuracy also has limitations. Larson et al. reported that the use of office endometrial biopsy had a sensitivity of 67%, specificity of 73% and accuracy of 70% for the diagnosis of G1 histopathology and that 37% of cases with G1 histopathology diagnosed by office endometrial biopsy were upgraded to G2/G3 adenocarcinoma after examination of specimens obtained from hysterectomy [10]. However, the level of accuracy for diagnosis of histologic grade would be higher and the number of upgraded cases would decrease if the category of diagnosis including both G1 and G2 is investigated. Larson et al. reported that the use of endometrial biopsy had an accuracy of 86% for the diagnosis of G1/G2 histopathology and that only 1.7% of cases with G1/G2 histopathology diagnosed by office endometrial biopsy were upgraded to G3 adenocarcinoma after examination of specimens obtained from hysterectomy [10].

According to our LNM score, the rate of para-aortic LNM in the low risk group was 0.5%. If treatment strategy including para-aortic lymphadenectomy rescued all patients with para-aortic LNM, the results shown in Fig. 2 suggest that performing para-aortic lymphadenectomy in all cases of endometrial cancer would result in improvement of the 5-year survival rate by about 10%. On the other hand, only 0.5% of patients with no risk factor would benefit from para-aortic lymphadenectomy. According to our

LNM score, the rate of LNM in the low risk group with histologic grade 1 and with invasion of less than half of the myometrium as assessed by MRI was only 1.7%. The rate of LNM increases in proportion to the number of resected lymph nodes [11]. Conversely, cases false-negative for LNM will increase if there are only a few resected lymph nodes. Since the number of resected lymph nodes in this study was much more than that in previous studies, the reported rates of LNM may be reliable.

To conclude, our LNM score may offer useful information for stratification of risk of LNM, and our results suggest that para-aortic lymphadenectomy can be eliminated in cases with no risk factors for LNM in the LNM score and that lymphadenectomy itself may be eliminated in some cases with no risk factors for LNM in the LNM score.

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Progesterone receptor isoforms as a prognostic marker in human endometrial carcinoma

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The possible role of specific progesterone receptor (PR) isoforms (PRA and PRB) as predictive factors in endometrial carcinoma is unclear. The present study was undertaken to evaluate the clinical significance of intratumoral PR isoform status in patients with endometrioid endometrial carcinoma. We studied 103 cases of endometrioid endometrial carcinoma using immunohistochemistry. We correlated the findings with various clinicopathological parameters of the patients. PRA and PRB immunoreactivity was detected in 51/103 (48.5%) and 79/103 (76.7%) of carcinoma cases, respectively. A significant positive correlation was detected between the status of PRB immunoreactivity and the amount of PRB mRNA by real-time reverse transcription-polymerase chain reaction ($P = 0.012$). PR isoform expression was significantly lower in the cases with higher histological grade ($P = 0.0001$ and $P = 0.002$, for PRA and PRB, respectively). Cases that were negative for either one or both PR isoforms were significantly associated with shorter disease-free and overall survival of the patients. The absence of either one or both of these two PR isoforms was detected in all nine patients who died (100.0%), whereas the absence of these immunoreactivities was detected only in 43 of 94 (45.7%) patients who had lived during the same period. In addition, multivariate analysis demonstrated that an absence of PRA immunoreactivity was an independent risk factor in disease-free survival of the patients ($P = 0.0258$). The results of our study demonstrated that loss or absence of PR isoform expression determined by immunohistochemistry could become an important prognostic indicator in patients with endometrioid endometrial carcinoma. (*Cancer Sci* 2006; 97: 1308–1314)

Endometrial carcinoma is one of the most common malignancies of the female genital tract and its incidence, especially that of endometrioid endometrial carcinoma, has increased recently.⁽¹⁾ It is well known that uterine endometrial proliferation is under the control of both estrogen and progesterone. One of the physiological roles of progesterone in the regulation of glandular epithelium of the endometrium is to induce cellular differentiation and to antagonize estrogen-mediated cell proliferation.⁽²⁾ Endometrial carcinogenesis is strongly associated with continued estrogen exposure without progesterone influence.^(3,4) Progesterone has clinically been demonstrated to provide some protection against stimulatory effects of estrogenic agents. In addition, hormone replacement therapy using combinations of estrogens and progesterones yields a lower risk of endometrial carcinoma, despite increasing the incidence of breast carcinoma.^(5,6) A number of the patients who wished to preserve their fertility were treated with progestin as a primary endocrine therapy for atypical hyperplasia and well-differentiated adenocarcinoma, although the effects of this treatment on the clinical outcome of patients have not always been satisfactory.^(7–9)

Both estrogen and progesterone act through intranuclear receptors, estrogen receptors (ER) and progesterone receptors (PR), which belong to the superfamily of steroid hormone receptors.⁽¹⁰⁾ The expression of ER and PR is generally considered to be coordinated because transcription of the PR gene is

induced by estrogen and inhibited by progesterones in the great majority of estrogen-responsive cells.⁽¹¹⁾ In normal cycling human endometrium, PR is expressed abundantly in glandular epithelium during the proliferative phase of the cycle.⁽¹²⁾ PR is present in two isoforms, termed PRA and PRB.⁽¹³⁾ PRA is the truncated form of PRB, lacking 164 amino acids at the NH₂ terminus. These isoforms are translated from the same gene, but transcription is initiated from different promoters.⁽¹⁴⁾ Studies addressing the individual effects of PR isoforms have been reported. Vegeto *et al.* reported that PRA could repress PRB activity in cells in which PRA was not transcriptionally active, and that PRA might be associated with a cell- and promoter-specific repressor of PRB.⁽¹⁵⁾ Giangrande *et al.* also reported that differential cofactor binding resulted in the opposing transcriptional activities of PRA and PRB.⁽¹⁶⁾ In addition, microarray analyses of human breast cancer cells expressing either PRA or PRB have confirmed that each PR isoform has a unique set of target genes, with little overlap.⁽¹⁷⁾ These functional and transcriptional differences suggest that the development, invasiveness and metastatic potential of carcinoma cells can be influenced by the PR status of the tumor cells. We previously reported that loss of PRB was a significant prognostic factor in epithelial ovarian cancer.^(18,19) In addition, breast carcinoma patients with PRA-rich tumors are in general associated with poorer disease-free survival rates.⁽²⁰⁾ In endometrial carcinoma, several studies demonstrated the PR isoform status of carcinoma cells.^(21–23) Arnett-Mansfield *et al.* reported a reduced expression of either one or both of the PR isoforms in the great majority of endometrial tumors, compared with hyperplastic or normal endometrium.⁽²¹⁾ De Vivo *et al.* demonstrated a polymorphism in the PRB promoter, which results in increased transcription of the PRB isotype. In a population-based study, this polymorphism was reported to be associated with increased risk for endometrial carcinoma.⁽²²⁾ In addition, hypermethylation of PRB alleles was detected in endometrial carcinoma.⁽²³⁾

Results of previous studies demonstrated that high levels of ER and PR were directly correlated with a lower tumor grade, less myometrial invasion, and a lower incidence of lymph node metastases in the patients with endometrioid endometrial carcinoma.^(24–27) In addition, the status of ER and PR in these carcinomas has been reported as an independent prognostic factor of the patients.⁽²⁸⁾ However, it is also true that there are many controversies regarding the possible roles of specific PR isoforms as predictive factors in endometrial carcinoma.^(21,29–32) Fujimoto *et al.* reported that PRA could not be detected in advanced endometrial tumors.⁽²⁹⁾ In accordance with this, they later reported that PRB was expressed predominantly in distant metastases of endometrial carcinoma.⁽³⁰⁾ In contrast, Kumar *et al.* reported that downregulation of PRB may be associated with poorly differentiated endometrial carcinoma.⁽³¹⁾ Sakaguchi *et al.*

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also proposed that the drastic decrement of PRB but not of PRA resulted in poor prognosis in endometrial carcinoma, although histological type was not described in their study.⁽³²⁾

Therefore, in the present study, we carried out immunohistochemical analysis of 103 cases of endometrioid endometrial carcinoma, and correlated the findings with the clinicopathological features of the patients, including their clinical outcome, in order to study the possible roles and correlation between PR isoforms and prognosis of the patients.

Materials and Methods

Endometrial carcinoma patients and tissue preparation. One hundred and three endometrioid endometrial carcinomas (49 well differentiated, 32 moderately differentiated, 22 poorly differentiated; 66 stage I, 12 stage II, 22 stage III, 3 stage IV) were retrieved from the surgical pathology files of Tohoku University Hospital, Sendai, Japan. The protocol for this study was approved by the Ethics Committee at Tohoku University School of Medicine (Sendai, Japan). None of the patients examined had received irradiation, hormonal therapy or chemotherapy prior to surgery. The median follow-up time of the patients examined in this study was 60 months (range, 2–148 months). The disease-free and overall survival times of the patients were calculated from the time of initial surgery to recurrence or death, or the date of last contact. The survival times of patients still alive or lost to follow-up were censored in December 2004. The clinicopathological findings of the patients, including age, histology, stage, grade and preoperative therapy, were retrieved by extensive review of the charts. A standard primary treatment for endometrial carcinoma at Tohoku University Hospital was surgery consisting of total abdominal hysterectomy, salpingo-oophorectomy, pelvic and/or para-aortic lymphadenectomy and peritoneal washing cytology. Eighty-five out of 103 patients (83%) in this study underwent complete surgery as above. Six out of 85 patients had lymph node metastasis. The remaining 18 patients (17%) underwent total abdominal hysterectomy and salpingo-oophorectomy without lymphadenectomy because of obesity or their poor performance status. The lesions were classified according to the Histological Typing of Female Genital Tract Tumors by WHO and staged according to the International Federation of Gynecology and Obstetrics system.^(33,34) Sixty-eight out of 103 patients received pelvic radiation therapy (50 Gy) or three to six courses of chemotherapy, consisting of the cisplatin-based combination regimen CAP (60–70 mg/m² cisplatin, 40 mg/m² doxorubicin and 500 mg/body cyclophosphamide) after operation. Patients who had early stage and low-grade disease (stage IA, G1, stage IA, G2 and stage IB, G1) and patients who were associated with poor performance status did not receive any adjuvant therapy. None of the patients received hormone therapy after operation. All specimens were processed routinely (i.e. 10% formalin fixed for 24–48 h, paraffin embedded, and thin sectioned [3 µM]).

Antibodies. Monoclonal antibodies for PRA (hPRA7) and PRB (hPRA2) were purchased from NeoMarkers (Fremont, CA, USA). The PRA (hPRA7) antibody used in this study recognized both PRA and PRB in immunoblot analysis.⁽³⁵⁾ However, Mote *et al.* reported that hPRA7 did not recognize PRB on immunohistochemistry in fixed tissues even after antigen retrieval, as evidenced by the absence of immunostaining by this antibody of the PRB-expressing MDA-MB-231/PRB cell line.⁽³⁶⁾ This was considered to be due to the inaccessibility of the epitope on PRB recognized by hPRA7 in 10% formalin-fixed and paraffin-embedded tissue specimens, possibly due to alteration of the conformation of the molecule in which the hPRA7 epitope is located in such a way to reduce its accessibility in immunohistochemistry. hPRA2 recognizes PRB exclusively.^(35,37) Monoclonal antibodies for ER α , ER β and Ki67 were purchased

from Novocastra (Benton, NC, UK), Genetex (San Antonio, TX, USA) and DAKO Cytomation (Carpinteria, CA, USA), respectively.

Immunohistochemistry. Immunostaining was carried out by the streptavidin–biotin amplification method using a Histofine Kit (Nichirei, Tokyo, Japan). Antigen retrieval was carried out using an autoclave treatment for 5 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate, pH 6.0). The dilutions of the primary antibodies used in our study were as follows: PRA, 1/100; PRB, 1/100; ER α , 1/50; ER β , 1/1500; and Ki67, 1/50. The antigen–antibody complex was visualized with 3,3'-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris-HCl buffer [pH 7.6] and 0.006% H₂O₂), and counterstained with hematoxylin. Proliferative-phase endometrial glands were used as positive controls for immunohistochemistry of PR isoforms⁽²⁶⁾ and breast cancers were used as positive controls for ER α and ER β . As a negative immunostaining control, normal rabbit or mouse IgG was used instead of the primary antibodies. No specific immunoreactivity was detected in these tissue sections.

Scoring of immunoreactivity. Evaluation of PRA, PRB, ER α , ER β and Ki-67 was carried out in high-power fields ($\times 400$) using a standard light microscope. Two of the authors (SS and KI) searched all of the tissue sections simultaneously and determined the most representative areas using a double-headed light microscope. In all of the cases examined, a total of more than 500 tumor cells from three different representative fields were counted independently by the two authors, and the percentage of immunoreactivity (i.e. the labeling index [LI]) was determined. After completely reviewing the immunostained sections of each lesion, two of the authors (SS and KI) independently divided the cases into the following two groups: +, >10% positive cells; and -, <10% positive cells. Layfield *et al.* proposed the separation of ER- and PR-positive cases using LI cut-off points of 10% in the immunohistochemical analysis of human breast cancer.⁽³⁸⁾ The eighth St Gallen meeting also recommended that approximately 10% positive staining of cells for either ER or PR might be considered as a reasonable threshold for definite endocrine responsiveness.⁽³⁹⁾ Therefore, in the present study, we used the same cut-off point of 10% between positive and negative PR isoforms, based on the results of the studies above. Cases with discordant results (interobserver differences of >5%) were reevaluated simultaneously the two authors above using a double-headed light microscope. Consequently, the interobserver differences were less than 5% in this study.

Reverse transcription–polymerase chain reaction. Thirty-three specimens of fresh frozen tissues of endometrial carcinoma (i.e. specimens frozen immediately in liquid nitrogen and stored at -80°C) were available for the present study. Total RNA was extracted by homogenizing frozen tissue samples in 1 mL TRIzol reagent (Life Technologies, Gaithersburg, Grand Island, NY, USA), followed by phenol–chloroform extraction and isopropanol precipitation. All RNA samples were quantified by spectrophotometry and stored at -80°C until processing for reverse transcription (RT). Total RNA (4 µg) was denatured at 70°C for 10 min and was reverse transcribed in the presence of 50 ng/µL Oligo (deoxythymidine) primer (Invitrogen, Carlsbad, CA, USA), 2.5 mmol/L MgCl₂, 0.5 mmol/L deoxy-NTPs, 10 mmol/L dithiothreitol and 10 IU ribonuclease H-reversed transcriptase (Superscript II RT, Invitrogen) for 60 min at 42°C and 15 min at 70°C on a PTC-200 Peltier Thermal Cycler DNA Engine (MJ Research, Watertown, MA, USA). RT–polymerase chain reaction (PCR) analysis was carried out in order to examine the presence or absence of genomic DNA contamination. The RT step was performed in the absence of Superscript II RNase H-reverse transcriptase, followed by PCR. RT-PCR products lacking reverse transcriptase in the initial RT step were run on an ethidium-bromide-stained 2% agarose gel. No bands were detected in these samples (data not shown). After an initial

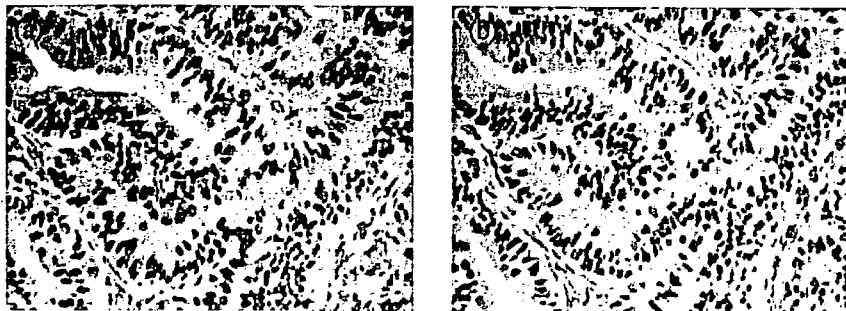


Fig. 1. Immunohistochemical staining for (a) progesterone receptor A (PRA) and (b) progesterone receptor B (PRB) in endometrioid endometrial carcinoma. PRA and PRB immunoreactive proteins were detected in the nuclei of carcinoma cells of G1 adenocarcinoma. Original magnification, $\times 400$.

1 min denaturation step at 96°C, 35 cycles of PCR were carried out on thermal cycle under the following conditions: 45 s denaturation at 94°C, 30 s annealing at 58°C, and a 1.5 min extension at 72°C. In addition, cDNA was used as a template for real-time PCR. Real-time PCR was carried out with the LightCycler System (Roche Diagnostics, Mannheim, Germany) using the DNA-binding dye SYBER Green I (Roche Diagnostics). The 20- μ L reaction mixture contained 3 mM MgCl₂, for PRB and β -actin primer, 10 pmol/L of each primer and DNA-binding dye LightCycler-Fast Start DNA Master SYBR Green I. β -Actin expression was used to verify the integrity of RNA from each specimen. Human gene-specific primers used to amplify PRB and β -actin were as follows: PRB 5' sense, ACACCTTGCC-TGAAGTTTCG and PRB 3' antisense, CTGTCCTTTTCTGG-GGGACT (196 bp); β -actin 5' sense, CCAACCGCGAGAA-GATGAC and β -actin 3' antisense, GGAAGGAAGGCTGG-AAGAGT (459 bp). An initial denaturing step at 95°C for 10 min was followed by 35 cycles of 95°C for 15 s, 10 s annealing at 58°C (PRB) and 63°C (β -actin), and extension for 13 s at 72°C. The fluorescence intensity of the double-strand-specific SYBER Green I, which reflects the amount of specific PCR products formed, was read by the LightCycler at 85°C after the end of each extension step.⁽⁴⁰⁾ Using automated programs of the LightCycler software, the amount of PRB and β -actin template in each sample was calculated so as to dilute the standard cDNA equally. The actual values of PRB were corrected by the value of the β -actin template. Although conventional quantitative PCR requires the use of purified plasma cDNA in the construction of a standard curve, it was possible to semiquantify the PCR products with the LightCycler using purified cDNA of known concentrations.^(41,42) In initial experiments, PCR products were purified and subjected to direct sequencing (ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit and ABI PRISM 310 Genetic Analyzer; Perkin-Elmer PE Applied Biosystems, Foster City, CA, USA) to verify amplification of the correct sequences. Frozen breast cancer tissue was used as a positive control. Negative control experiments did not contain cDNA substrate to study the presence of exogenous contamination of DNA. No amplified products were detected under these conditions.

Statistical analyses. Statistical analysis was carried out using SAS software (StatView, Version 5.0; SAS, Cary, NC, USA). The statistical significance of the association between PRA and PRB immunoreactivity and other parameters (grade, stage, age, ER α LI, ER β LI and Ki-67 LI) was evaluated using the Mann-Whitney *U*-test and the χ^2 -test. The statistical significance between PRA and PRB immunoreactivity was calculated using a correlation coefficient (*r*) and regression equation. The statistical significance between PRB immunoreactivity determined by immunohistochemistry and the status of mRNA determined by RT-PCR was evaluated using Fisher's exact probability test, and the statistical significance between PRB immunoreactivity and amounts of PRB mRNA determined by real time RT-PCR was evaluated using the Mann-Whitney *U*-test. The Kaplan-Meier method and statistical significance was calculated using a log-

rank test. Univariate and multivariate analyses were evaluated using Cox's proportional hazards model. *P*-values less than 0.05 were considered significant.

Results

Immunohistochemistry and RT-PCR. Immunoreactivity for PRA and PRB was detected in the nuclei of carcinoma cells (Fig. 1). ER α , ER β and Ki-67 were also confined exclusively to the nuclei of epithelial cells (data not shown). RT-PCR was carried out to confirm the expression of PRB using 33 cases in this study (Figs 2,3), because PRA has no specific sequence to distinguish it from PRB mRNA by RT-PCR. Twenty-five of these 33 cases were PRB positive and eight cases were PRB negative, as determined by immunohistochemistry. PRB mRNA was detected in 21 out of these 25 PR-positive cases (84%) and was not detected in five out of eight PRB-negative cases (Fig. 2). There was a statistically significant positive correlation between PRB immunoreactivity and mRNA expression examined by RT-PCR analysis (*P* = 0.02). In addition, amounts of PRB mRNA determined by real time RT-PCR were 8.89 (median values) in these PRB-positive and 0.41 (median values) in PRB-negative cases. A significant positive correlation was detected between PRB immunoreactivity and the amounts of PRB mRNA (*P* = 0.012) (Fig. 3). Eighty out of 103 cases (77.7%) demonstrated either or both PR isoforms in immunohistochemistry. Fifty-one out of 103 cases (48.5%) were PRA positive. Among these 51 PRA-positive cases only one case (1.9%) was PRA positive and PRB negative. However, PRB-positive cases were 76.7% (79/103), and 29 of these 79 PRB-positive cases (36.7%) were both PRB positive and PRA negative. The proportion of cases positive for both PRA and PRB was 48.5% (50/103), whereas the proportion of cases negative for both PRA and PRB was 22.3% (23/103). There was a significant positive correlation between PRA and PRB expression in endometrial carcinoma (*P* = 0.004). Results of the associations between clinicopathological parameters and immunoreactivity of PRA and PRB are summarized in Table 1. The status of PRA in G1, G2 and G3 endometrial carcinoma was 67.3% (33/49), 46.6% (15/32) and 13.6% (3/22), respectively, and the status of PRB was 87.8% (43/49), 78.1% (25/32) and 50.0% (11/22), respectively. PR immunoreactivity was significantly lower for carcinoma with higher histological grade (*P* = 0.0001 and *P* = 0.002, for PRA and PRB, respectively), whereas there were no correlation among the clinical stages of the cases. PRA and PRB expression was significantly positively correlated with ER α LI, and inversely with Ki-67 LI.

Relationship between PR isoform expression and prognosis. Progesterone receptor isoform status was evaluated as a prognostic variable in the patients with endometrioid endometrial carcinoma using univariate analysis. Results of univariate analysis are summarized in Table 2. The following variables were significantly associated with poorer disease-free survival and overall survival of the patients at the *P* < 0.05 levels: absence of PRA immunoreactivity; absence of PRB immunoreactivity;



Fig. 2. Reverse transcription-polymerase chain reaction (RT-PCR) analysis of total RNA extracted from endometrioid endometrial carcinoma. Nos 4, 10, 13, 21, 29, 30, 31, 32 and 33 are progesterone receptor B (PRB)-negative cases, as determined by immunohistochemistry. No. 34 is a positive control. No. 35 is a negative control. PRB mRNA was detected in 21 out of these 25 PR-positive cases (84%) and not detected in five out of eight PR-negative cases. There was a statistically significant positive correlation between PRB immunoreactivity and mRNA expression examined by RT-PCR analysis ($P = 0.02$, Fisher's exact probability test).

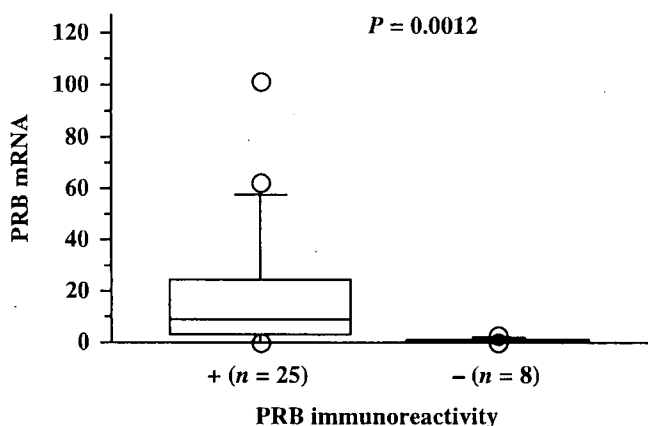


Fig. 3. Correlation between progesterone receptor B (PRB) immunoreactivity and its mRNA level determined by quantitative reverse transcriptase-polymerase chain reaction analyses in human endometrial carcinoma. There was a statistically significant positive correlation between PRB immunoreactivity and the amount of PRB mRNA ($P = 0.012$, Mann-Whitney U -test).

and histological grades. The disease-free and overall survival curves of the patients according to the Kaplan-Meier method are demonstrated in Fig. 2. The 5-year disease-free and overall survival rates were 95.6% and 96.4%, respectively, for PRA-positive cases and 71.1% and 84.3%, respectively, for PRA-negative cases. Patients with negative PRA in these carcinoma tissues were associated with a significantly poorer prognosis than those of PRA-positive cases at both disease-free ($P = 0.0009$) and overall survival ($P = 0.0098$) (Fig. 2A,B). Fig. 2 also demonstrates the greater disease-free and overall survival

of the PRB-positive cases compared to PRB-negative cases ($P = 0.0007$ and $P = 0.0116$, respectively). The 5-year disease-free and overall survival times were 90.5% and 94.1%, respectively, for PRB-positive cases and 61.3% and 75.9%, respectively, for PRB-negative cases. In addition, the absence of either one or both of these two PR isoforms was associated with a significantly poorer prognosis at disease-free survival ($P = 0.0005$) (Fig. 2C). In addition, the absence of either one or both of these two PR isoforms was detected in all nine patients who died (100.0%), whereas the absence of these immunoreactivities was detected only in 43 of 94 (45.7%) patients who lived during the same period.

In order to determine whether the prognostic value of PRA or PRB expression was independent of other risk factors associated with clinical outcome of the patients with endometrioid endometrial carcinoma, we examined the results using multivariate analysis. The prognostic factors examined were the status of PRA or PRB, ER, stages and histological grades. As shown in Table 3, absence of PRA in carcinoma tissue was statistically significant as an independent risk factor only in disease-free survival of the patients ($P = 0.0258$), although PRB status was not a significant factor in disease-free or overall survival. Histological grade turned out to be an independent risk factor only in overall survival of the patients.

Discussion

This is the first study demonstrating that the absence of not only PRA but also PRB expression determined by immunohistochemistry is an important prognostic indicator of patients with endometrioid endometrial carcinoma. Progesterone is known to be one of the very important endocrine factors regulating cellular proliferation of the endometrium and its effects are mediated through PR.⁽¹⁰⁾ PR has two isoforms, PRA and PRB, but the exact biological or clinical differences between the roles

Table 1. Correlation between progesterone receptor isoform A and B (PRA and PRB) immunoreactivity and clinicopathological parameters in endometrial carcinoma

Parameter	Total (n = 103)	PRA		P-value	PRB		P-value
		+	-		+	-	
		(n = 51)	(n = 52)		(n = 79)	(n = 24)	
Age (years)							
50	22	15	7		19	3	
>50	81	36	45	0.048	60	21	0.27
Grade							
1	49 (47.6%)	33	16		43	6	
2	32 (31.0%)	15	17		25	7	
3	22 (21.4%)	3	19	0.0001	11	11	0.002
Stage							
I, II	78 (75.7%)	40	38		63	15	
III, IV	25 (24.3%)	11	14	0.526	16	9	0.08
ER α LI (median)	23	34	11	0.003	34	4.5	< 0.0001
ER β LI (median)	5	5	8	0.3	11	2	0.089
Ki67 LI (median)	32	27	40	0.003	30	46	0.002

ER, estrogen receptor; LI, labeling index.

Table 2. Univariate analyses (P-values) of predictors of disease-free and overall survival for 103 patients with endometrial carcinoma

Variable	Disease-free survival	Overall survival
PRA (positive vs negative)	0.0055	0.0354
PRB (positive vs negative)	0.0022	0.0225
Age (\leq 50 years vs 50 years)	0.1159	0.0854
Stage (I/II vs III/IV)	0.2029	0.1163
Histological grade (1-3)	0.0276	0.0067
ER α (positive vs negative)	0.0426	0.2667
ER β (positive vs negative)	0.4832	0.3965
Ki67 (positive vs negative)	0.4722	0.3487

ER, estrogen receptor; PR, progesterone receptor.

of these two PR isoforms in endometrial carcinoma remains largely unknown. The results of our present study demonstrated that PRB was more common than PRA in endometrioid endometrial carcinoma, which is consistent with a recent report

by Miyamoto *et al.*⁽⁴³⁾ They reported PRB LI of 30.4%, whereas those of PRA were 11.3% in endometrial carcinoma. Sakaguchi *et al.* also reported that PRB expression was more common than PRA expression in endometrial carcinoma.⁽³²⁾ However, Arnett-Mansfield *et al.* reported that PRA, not PRB, was dominant in endometrial carcinoma.⁽²¹⁾ This discrepancy of results may be explained by the number of cases examined, because Arnett-Mansfield *et al.* examined a relatively small number of cases (46 cases), whereas our present study as well as others examined PR expression in more than 100 patients with endometrial carcinoma. We demonstrated previously that PRB was expressed dominantly in all types of epithelial ovarian cancer.^(18,19) In human breast cancer, however, PRA was dominant in invasive ductal carcinoma.^(20,44) Therefore, the biological significance of PR isoforms may differ depending on tumors, even among human estrogen-dependent carcinomas.

Progesterone receptor and ER are known to be among the most extensively studied biological prognostic markers in endometrial carcinoma. However, the status of PR isoforms and their possible roles in conjunction with clinical outcome in patients with endometrial carcinoma have not been fully

Table 3. Multivariate analyses of predictors of disease-free and overall survival for 103 patients with endometrial carcinoma

Predictor	Disease-free survival		Overall survival	
	HR (95% CI)	P-value	HR (95% CI)	P-value
PRA (positive vs negative)	0.171 (0.036-0.808)	0.0258	0.196 (0.022-1.764)	0.1522
Histological grade (1-3)	1.333 (0.728-2.440)	0.3514	2.371 (0.948-5.931)	0.065
ER α (positive vs negative)	0.509 (0.186-1.394)	0.1888	0.748 (0.187-2.992)	0.6818
Stage (I-IV)	0.374 (0.231-2.287)	0.1053	1.451 (0.785-2.685)	0.2352
PRB (positive vs negative)	0.37 (0.121-1.125)	0.0798	0.445 (0.102-1.932)	0.2797
Histological grade (1-3)	1.569 (0.852-2.888)	0.1481	2.838 (1.116-7.217)	0.0285
ER α (positive vs negative)	0.557 (0.192-1.610)	0.2798	0.794 (0.188-3.360)	0.9184
Stage (I-IV)	0.2191 (0.837-2.171)	0.2192	1.387 (0.730-2.635)	0.3174

CI, confidence interval; ER, estrogen receptor; HR, hazard ratio; PR, progesterone receptor.

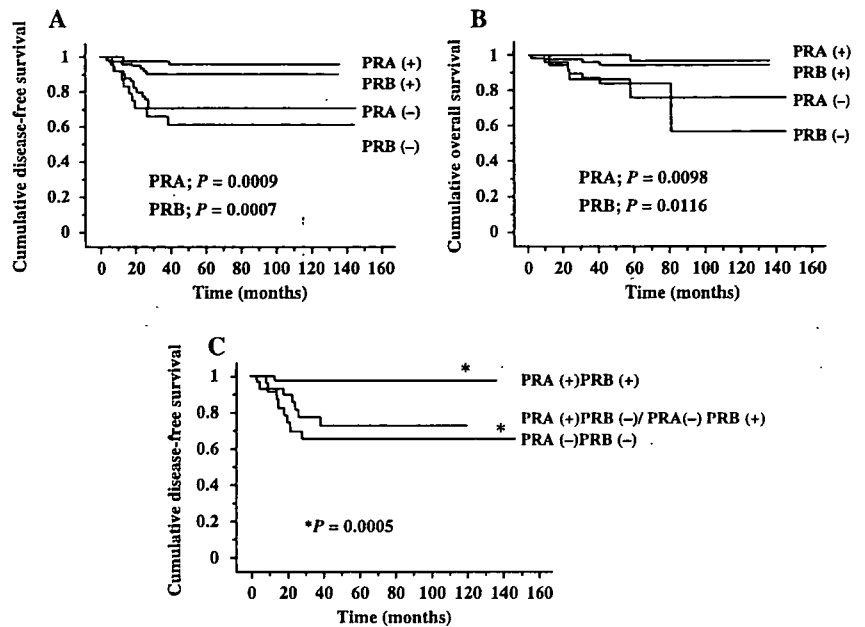


Fig. 4. Correlation between progesterone receptor A (PRA) or B (PRB) isoform immunoreactivity and (A) recurrence, and (B) survival for patients with endometrioid endometrial carcinoma. (C) Correlation between immunoreactivity for both isoforms and recurrence for patients with endometrioid endometrial carcinoma.

characterized. There have been some reports demonstrating the status of PR isoforms and clinical prognosis in endometrial carcinoma.^(32,43) Miyamoto *et al.* carried out immunohistochemical analysis and demonstrated that PRB expression occurred significantly more frequently in grade 1 and was inversely correlated with poor prognosis on clinical outcome of patients, whereas PRA expression was also significantly higher in grade 1 and was inversely correlated with Ki-67 expression, but not with prognosis of the patients. They concluded that PRA and PRB expression was significantly correlated with biologically malignant potential.⁽⁴³⁾ Sakaguchi *et al.* examined mRNA levels of the PR isoforms and reported a significant positive correlation between PRA and PRB mRNA expression in endometrial carcinoma.⁽³²⁾ They quantified the mRNA levels of PRAB (PRA + PRB) using real-time RT-PCR, and they also calculated the mRNA levels of PRA from these data. There were no significant differences in the level of PRA mRNA between normal endometrium and each histological grade, although PRB expression was significantly higher in G1. In addition, PRB mRNA, but not PRA mRNA, status was significantly correlated with survival in endometrial carcinoma.⁽³²⁾ However, in these previous studies, the combined results for loss of expression of both of the PR isoforms and their prognostic correlations were not examined in endometrioid endometrial carcinoma. In the present study, both PRA and PRB were significantly lower for the higher histological-grade carcinoma cases, which is consistent with the results of previously reported studies.^(30,32,43) Loss of both PRB and PRA expression in carcinoma tissue was significantly associated with an adverse clinical outcome in the patients. The absence of either one or both of these PR isoforms was associated with a significantly poorer prognosis at disease-free survival. In addition, multivariate analysis demonstrated that an absence of PRA immunoreactivity was an independent risk factor in disease-free survival of the patients (Table 3). Furthermore, only one case was PRB negative among these 51 PRA-positive cases. The number and disease-free survival curve was similar between the groups of PRA⁻PRB⁻ and PRA⁺PRB⁻/PRA⁻PRB⁺ (Fig. 4C). These results all

indicate that the status of PRA in endometrial cancer is quite important in determining the postoperative course of the patients.

Each PR status is considered to strongly influence the abnormal proliferative, invasive and metastatic potential of endometrial carcinoma cells. Microarray analysis of human endometrial carcinoma cells expressing either PRA or PRB confirmed that each PR isoform has distinctly different target genes, with little overlap.⁽⁴⁵⁾ Several investigators demonstrated that progesterone acts principally through PRB to inhibit endometrial carcinoma cell invasiveness modulated by adhesion molecules, including integrin and matrix metalloproteinases.^(46,47) However, Hanekamp *et al.* demonstrated recently that endometrial carcinoma cell lines, which expressed only PRA, expressed higher levels of cadherins and demonstrated a lower level of invasive properties compared to the cell lines that expressed PRB.⁽⁴⁸⁾ They also demonstrated that the loss of expression of both PR isoforms was associated with increased expression of CD44 and CSPG/versican, invasion-related proteins. They further suggested that these results may represent an early and possibly initializing event in the development of a more invasive phenotype in endometrial carcinoma.⁽⁴⁹⁾ Results of these studies in cell lines also suggest that a decrease or loss of PRA and/or PRB expression should become an important factor that contributes to invasive and metastatic potential and eventually poor prognosis in human endometrial carcinoma. Dai *et al.* studied the effectiveness of adenovirus-mediated PR gene transduction in combination with progestin therapy in mouse xenograft models, and demonstrated that the presence of both PRA and PRB provided a substantial benefit to animal survival compared with PRB alone.⁽⁵⁰⁾ Results of an inverse correlation between both PR isoforms and Ki-67 expression in our study also suggest the important roles of each PR isoforms for protecting against aggressive proliferation and development. In summary, the results of the present study indicate that the loss of PR isoform expression, especially PRA, in human endometrioid endometrial carcinoma may result in aggressive biological characteristics that play important roles in prognosis and recurrence.

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Peroxisome Proliferator-Activated Receptor γ and Growth Inhibition by Its Ligands in Uterine Endometrial Carcinoma

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Abstract Purpose: In this study, we evaluated the correlation between endometrial carcinoma and peroxisome proliferator-activated receptor γ (PPAR γ) expression and assessed whether PPAR γ ligands influence carcinoma growth.

Experimental Design: We examined the presence and cellular distribution of PPAR γ protein in 42 normal endometria, 32 endometria with hyperplasia, and 103 endometria with endometrial carcinoma by immunohistochemistry. We then compared PPAR γ mRNA expression in endometrial carcinoma with that in normal endometria using real-time reverse transcription-PCR. We subsequently confirmed expression of PPAR γ mRNA by real-time reverse transcription-PCR and PPAR γ protein by immunoblotting in endometrial carcinoma cell lines (Ishikawa, Sawano, and RL95-2 cells). We further examined the effects of PPAR γ agonist 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15d-PGJ₂), a naturally occurring PPAR γ ligand, to these endometrial carcinoma cell lines. We also examined the status of apoptosis and p21 mRNA expression of these endometrial carcinoma cell lines following addition of 15d-PGJ₂.

Results: PPAR γ immunoreactivity was detected in 11 of 23 (48%) of proliferative-phase endometrium, 14 of 19 (74%) of secretory-phase endometrium, 27 of 32 (84%) of endometrial hyperplasia, and 67 of 103 (65%) of carcinoma cases. PPAR γ immunoreactivity was significantly lower in endometrial carcinoma than in secretory-phase endometrium ($P = 0.012$) and endometrial hyperplasia ($P = 0.006$). There was a significant positive association between the status of PPAR γ and p21 expression in endometrial carcinoma ($P < 0.0001$). There was a significant negative association between the body mass index and PPAR γ labeling index of carcinoma tissue in the patients with endometrial carcinoma ($P < 0.0001$). PPAR γ mRNA was expressed abundantly in normal endometria but not in endometrial carcinoma. We showed that PPAR γ agonist 15d-PGJ₂ inhibited cell proliferation and induced p21 mRNA of endometrial carcinoma cell lines.

Conclusion: We showed the expression of PPAR γ in human endometrial carcinoma and the effects of PPAR γ ligand in endometrial carcinoma cells. These findings suggest that a PPAR γ ligand, 15d-PGJ₂, has antiproliferative activity against endometrial carcinoma.

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Peroxisome proliferator-activated receptor (PPAR) is a member of the nuclear hormone receptor superfamily of transcription factors. PPARs function as transactivation factors following heterodimerization with retinoid X receptors (RXR) and bind to its specific response elements, termed peroxisome proliferator-responsive elements, of various target genes (1). PPARs have a subfamily of three different isoforms: PPAR α , PPAR β/δ , and PPAR γ . All isoforms play important roles in the regulation of several metabolic pathways, including lipid biosynthesis and glucose metabolism. There are distinctive differences in the distribution of these receptor subtypes in humans. PPAR α has been detected mainly in the myocardium, kidney, and liver; PPAR β/δ is expressed in most tissues; and PPAR γ has been found primarily in adipocytes and immune cells (2, 3).

PPAR γ plays important roles in the regulation of lipid homeostasis, adipogenesis, insulin resistance, and development of various organs (4–6). Naturally occurring PPAR γ ligand, a

15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15d-PGJ₂), activates PPAR γ at micromolar concentrations in human *in vivo* (7–9). Synthetic PPAR γ ligands are known as thiazolidinediones, including troglitazone, rosiglitazone, and pioglitazone. These synthetic ligands have been used for the treatment of insulin resistance in type II diabetes mellitus. In addition, thiazolidinediones have been proposed in differentiation-mediated therapy of various human carcinomas associated with high levels of PPAR γ (10).

Various *in vitro* studies showed that PPAR γ ligands have a potent antiproliferative activity for a wide variety of neoplastic cells (11). PPAR γ agonist was reported to inhibit the proliferation of carcinoma cells, and phase II clinical trials using PPAR γ ligands have recently been done as a novel therapy for advanced patients with breast carcinoma, histologically confirmed prostate carcinoma, liposarcoma, and metastatic colon carcinoma (12). The outcomes of these trials are, however, still controversial. Mueller et al. reported that one advanced prostate carcinoma patient had a dramatic decrease in serum prostate-specific antigen as a result of troglitazone treatment (13), but Burstein et al. reported troglitazone had little apparent clinical value among patients with treatment-refractory metastatic breast carcinoma (14). In addition, Debrock et al. reported no significant changes in the histologic appearance of the liposarcomas following treatment with rosiglitazone (15).

The expression and effectiveness of PPAR γ has been extensively studied in breast, prostate, and colon carcinoma, but little is known about PPAR γ in uterine endometrial carcinoma. In addition, obesity, excess estrogen, type II diabetes, and hypertension are some of the important risk factors of endometrial carcinoma (16–19), but the effects of PPAR γ agonists to endometrial carcinoma are largely unknown. Therefore, in this study, we first examined the expression of PPAR γ in endometrial carcinoma, correlated the findings, including the status of progesterone receptor, estrogen receptor α (ER α), ER β , Ki-67, and p21 and the body mass index (BMI) of the patients, and examined the growth inhibition of the carcinomas by PPAR γ agonists *in vitro* to clarify the possible biological and clinical roles of PPAR γ in human endometrial malignancy.

Materials and Methods

Patients and tissues. All patients were informed of the purpose of this study, and informed consent was obtained before participation. The research protocol was approved by the Ethics Committee of Tohoku University Graduate School of Medicine. Uterine endometrial carcinoma tissues (49 well differentiated, 32 moderately differentiated, and 22 poorly differentiated; 66 stage I, 12 stage II, 22 stage III, and 3 stage IV) were obtained from 103 patients who underwent surgery at the Department of Obstetrics and Gynecology, Tohoku University Hospital (Sendai, Japan) from 1993 to 2004. All the subjects were Japanese. All endometrial carcinoma specimens were obtained after hysterectomy. Median follow-up time of the patients examined in this study was 60 months (range, 2–148 months). Disease-free survival and overall survival were calculated from the time of initial surgery to recurrence and/or death or the date of last contact. Survival times of patients still alive or lost to follow-up were censored in December 2004. Information regarding age, BMI, stage, grade, primary surgery, postoperative therapy and recurrence, and complications were all

retrieved by a review of the chart. BMI was calculated by dividing weight in kilograms by the height in meters squared. We defined obesity in these patients as a BMI \geq 25 (20). A standard primary treatment for endometrial carcinoma at Tohoku University Hospital from 1993 to 2004 was total abdominal hysterectomy, salpingo-oophorectomy, pelvic and/or para-aortic lymphadenectomy, and peritoneal washing cytology. A total of 85 of 103 (83%) patients underwent complete surgery. Six of the 85 patients had lymph node metastasis. The remaining 18 (17%) patients underwent total abdominal hysterectomy and salpingo-oophorectomy without lymphadenectomy because of obesity and/or poor performance status. None of these patients had received preoperative chemotherapy and/or hormonal therapy or pelvic irradiation. None of the patients used oral contraceptives. The lesions were classified according to the Histological Typing of Female Genital Tract Tumors by WHO and staged according to the International Federation of Gynecology and Obstetrics system (21, 22). About 60% of 103 patients received pelvic radiation therapy (50 Gy) or three to six courses of chemotherapy consisting of the cisplatin-based combination regimen CAP (cisplatin 60–70 mg/m², doxorubicin 40 mg/m², and cyclophosphamide 500 mg/body weight) after operation. Patients who had early-stage and low-grade disease (stage IA, grade 1, stage IA, grade 2, and stage IB, grade 1) and patients who were associated with poor performance status did not receive any adjuvant therapy. All of the archival specimens were retrieved from the surgical pathology files at Tohoku University Hospital.

Normal endometrial tissues were obtained from unaffected endometrium of normally menstruating females who underwent hysterectomy for cervical carcinoma *in situ*. Biopsy specimens from 23 cases were in the proliferative phase, and 19 cases were in the secretory phase. Fourteen had simple hyperplasia, 9 had complex hyperplasia with atypia, and 8 had complex hyperplasia without atypia. All specimens were routinely processed (i.e., 10% formalin fixed for 24–48 hours), paraffin embedded, and thin sectioned (3 μ m).

Antibodies. Monoclonal antibodies for PPAR γ were purchased from Perseus Proteomics Co. Ltd. (Tokyo, Japan). This antibody recognizes both human PPAR γ 1 and PPAR γ 2. Monoclonal antibody for β -actin was purchased from Sigma-Aldrich Co. (St. Louis, MO), monoclonal antibody for progesterone receptor was purchased from Chemicon International, Inc. (Temecula, CA), monoclonal antibodies for ER α and Ki-67 were purchased from Dako Cytomation Co. Ltd. (Glostrup, Denmark), monoclonal antibody was purchased for ER β from Gene Tex, Inc. (San Antonio, TX), and monoclonal antibody was purchased for p21 from Pharmingen (San Diego, CA).

Immunohistochemistry. A Histofine kit (Nichirei, Tokyo, Japan), which employs the streptavidin-biotin amplification method, was used in this study. Antigen retrieval for PPAR γ immunostaining was done by heating the slides in an autoclave at 120°C for 5 minutes (p21; 15 minutes) in citric acid buffer [2 mmol/L citric acid, 9 mmol/L trisodium citrate dehydrate (pH 6.0)]. Dilution of antibodies used in this study was as follows: 1:300 for PPAR γ , 1:40 for progesterone receptor, 1:50 for ER α , 1:1,500 for ER β , 1:50 for Ki-67, and 1:250 for p21. The antigen-antibody complex was visualized with 3,3'-diaminobenzidine solution [1 mmol/L 3,3'-diaminobenzidine, 50 mmol/L Tris-HCl (pH 7.6), 0.006% H₂O₂] and counterstained with hematoxylin. As a positive control for immunohistochemistry of PPAR γ , normal mammary glands and omentum tissues were employed in this study. Immunohistochemical staining was evaluated by two independent observers (K.O. and T.S.) without knowledge of clinical outcomes. PPAR γ immunoreactivity was detected in the nucleus, and the immunoreactivity was quantitated as the labeling index (LI). Five hundred carcinoma cells in each field were selected and the LI was determined as the percentage of positive cells per 500 carcinoma cells. For each tissue section, at least three fields were photographed under light microscopy at \times 200. Cases with a PPAR γ LI of $>$ 10% were considered PPAR γ -positive endometrial carcinoma in this study. Interobserver and intraobserver differences were $<$ 5% in our present study.

Table 1. Complications in endometrial carcinoma patients

	Endometrial carcinoma (n = 101), n (%)
Obesity (BMI \geq 25)	
+	45 (44.6)
-	56 (55.4)
Hypertension	
+	56 (55.4)
-	45 (44.6)
Diabetes mellitus (type II)	
+	24 (23.8)
-	77 (76.2)
Hyperlipidemia	
+	14 (13.9)
-	87 (86.1)
Breast carcinoma	
+	5 (5.0)
-	96 (95.0)

Cell lines and media. Uterine endometrial carcinoma cell lines, Ishikawa 3-H-12, Sawano, and RL95-2, were cultured in phenol red-free RPMI 1640 (Sigma-Aldrich) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS) and 1% penicillin/streptomycin. Ishikawa cells were originally established from well-differentiated human endometrial carcinoma (23). Sawano cells were naturally raised cisplatin-resistant cells (24). RL95-2 cells are ER-positive endometrial carcinoma cell line.

Real-time PCR. Total RNA from tissues, Ishikawa, Sawano, and RL95-2, were extracted using TRIzol reagent (Invitrogen Life Technologies, Inc., Gaithersburg, MD), and reverse transcription kit [SuperScript II Pre-amplification System (Gibco-BRL Invitrogen Co., Carlsbad, CA)] was used in the synthesis of cDNA. The LightCycler System (Roche Diagnostics GmbH, Mannheim, Germany) was used to semiquantify the mRNA expression levels using real-time PCR. The primer sequences used in this study were as follows: PPAR γ 5'-ATGCTGGCTCCTTGATCAATAA-3' and 5'-AGGGCTTGTAGCAGGTTGTCTTG-3' (266 bp), RXR α 5'-TTCGCTATGCTCTCAG-3' and 5'-ATAAGGAAGGTGTC AATGGG-3' (113 bp), RXR β 5'-GAAGCTCAGGCAAACTAC-3' and 5'-TCTTGTGTGCCC-3' (111 bp), RXR γ 5'-GCAGTTCAGAGGACATCAAGCC-3' and 5'-GCCTCACTCTCAGCTCGCTCTC-3' (352 bp), ribosomal protein L 13a 5'-CCTGGAGGAGAAGAGAAAGAGA-3' and 5'-TTGAGGACCTCTGTATTTGTCAA-3' (125 bp); and p21 5'-GGAAGACCATGTGGACCTGT-3' and 5'-GGATTAGGGCTTCCTCTTGG-3' (178 bp). Settings for the PCR thermal profile were initial denaturation at 95°C for 1 minute followed by 40 amplification cycles of 95°C for 1 second, annealing at 58°C (RXR α), 62°C (PPAR γ), 60°C (RXR β and p21), 68°C (ribosomal protein L 13a and RXR γ) for 15 seconds, and extension at 72°C for 15 seconds. To verify amplification of the correct sequences, PCR products were purified and subjected to direct sequencing. Negative control experiments lacked cDNA substrate to check for the possibility of exogenous contaminant DNA. Fat tissue cDNA was used as a positive control. The mRNA levels were summarized as the ratio of ribosomal protein L 13a and subsequently evaluated as a ratio (%) compared with that of controls. The PCR products were separated electrophoretically on a 2% agarose gel and stained with ethidium bromide.

Immunoblotting. Cells were grown to 70% confluence in 10-cm plates, and after removal of culture medium with PBS, nuclear protein was extracted by conventional method. The protein concentration was measured by a Model 680 microplate reader (Bio-Rad, Hercules, CA) using Bradford reagent (Bio-Rad). In all, 60 μ g nuclear protein of each

sample was mixed with an equal volume of 2 \times concentrated SDS-PAGE sample buffer, boiled, and then transferred to nitrocellulose membrane (Hybond polyvinylidene difluoride, Bio-Rad). The membranes were incubated in blocking solution (PBS containing 3% nonfat milk and 0.1% Tween 20) and then incubated in 1:500 dilution of PPAR γ antibody in PBS (containing 0.1% Tween 20) overnight at 4°C. After incubation with horseradish peroxidase-labeled anti-mouse IgM (Santa Cruz Biotechnology, Santa Cruz, CA), the antigen-antibody complex was visualized with enhanced chemiluminescence system (Amersham, Freiburg, Germany). β -Actin (Sigma-Aldrich) was used as internal positive control, and the dilution was 1:1,000.

Cell proliferation assay and apoptosis analysis. Cell proliferation was assessed by the Cell Counting Kit-8 (Wako Pure Chemical Industries, Osaka, Japan). We also examined the status of apoptosis in Ishikawa, Sawano, and RL95-2 cells using an apoptosis screening kit (Wako), which employed a modified terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling method (25). Ishikawa, Sawano, and RL95-2 cells were plated at 5×10^3 per well in 96-well plates and cultured with phenol red-free RPMI 1640 containing 10% dextran-coated, charcoal-stripped fetal bovine serum at 37°C for 2 days in a CO $_2$ incubator. 15d-PGJ $_2$ was purchased from Biomol Research Laboratories, Inc. (Butler Pike, PA), dissolved in ethanol, and added to the wells (day 0), and the assay was done on days 0, 1, 3, and 5. Control cells were treated with vehicle only. For cell proliferation assay, WST-1 solution was added to each well of the plates and incubated at 37°C for 2 hours. The absorbance at 450 nm was measured in a microplate reader SpectraMax 190 (Molecular Devices Corp., Osaka, Japan). The cell number and apoptosis index were calculated according to the following equation: (cell absorbance value after test materials treated / vehicle control cell absorbance value) and were subsequently evaluated as a ratio (%) compared with that of controls.

Statistical analysis. The statistical analysis was done using StatView 5.0 (SAS Institute, Inc.) software. An association between immunoreactivity for PPAR γ and clinicopathologic factors, results of cell proliferation assay, and results of real-time PCR were evaluated using Bonferroni/Dunn test, Mann-Whitney U test, or Wilcoxon test. Overall and disease-free survival curves were generated according to the Kaplan-Meier method and the statistical significance was calculated using the log-rank test. Results were considered significant when the $P < 0.05$.

Results

Complications of endometrial carcinoma patients. The median BMI for carcinoma patients was 24.6 (range, 17.3-39.6). BMI \geq 25 was 45 (44.6%) of endometrial carcinoma patients examined in our present study (Table 1). The number of

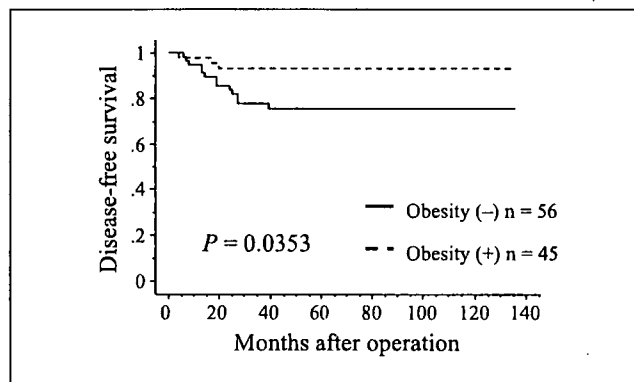


Fig. 1. Disease-free survival in endometrial carcinoma patients. Patients with obesity had longer disease-free survival.

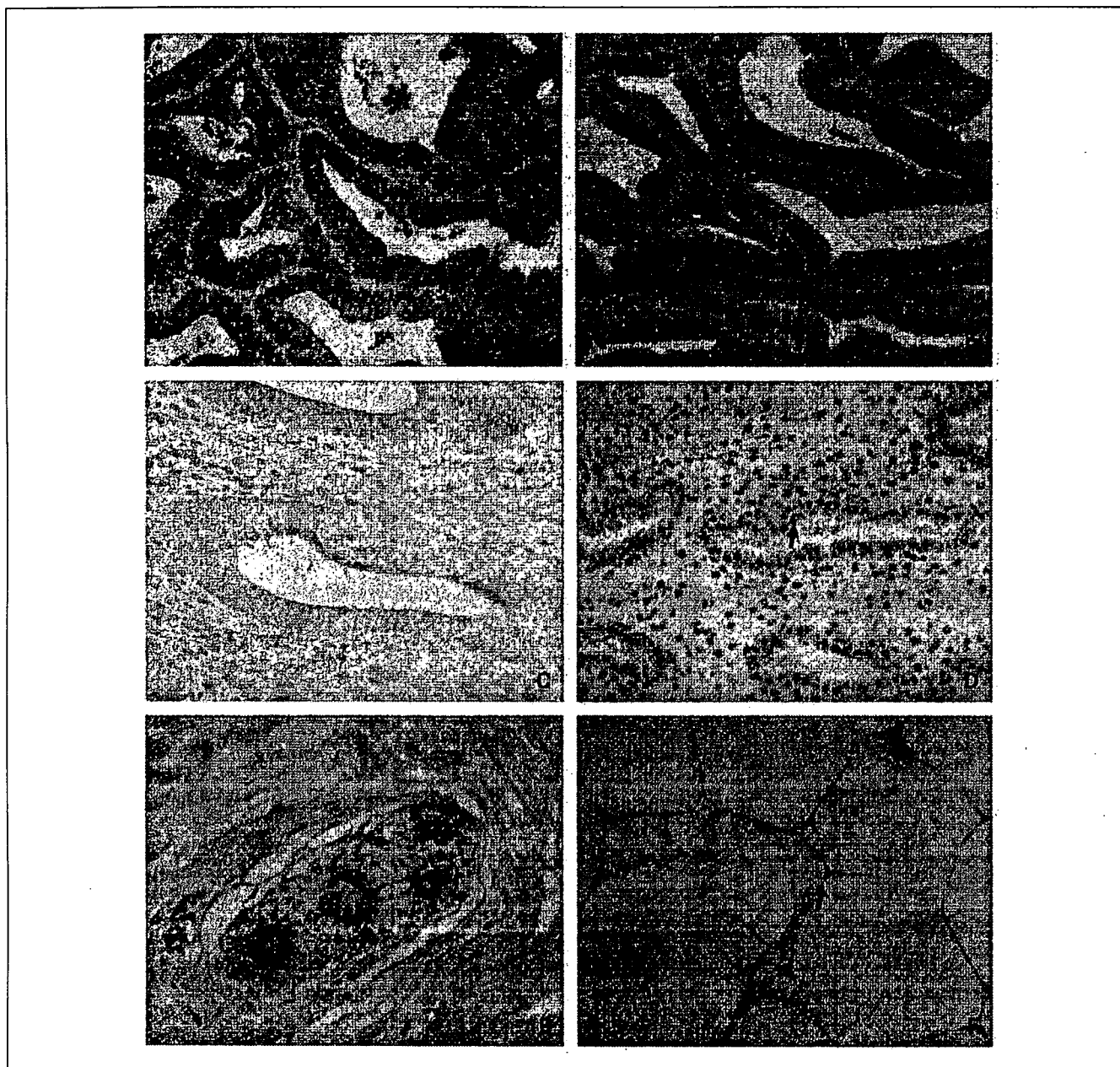


Fig. 2. Immunohistochemistry for PPAR γ and p21 ($\times 200$). Arrows, immunoreactive cells. *A*, PPAR γ immunoreactivity was detected in the nuclei of the endometrial carcinoma cells. *B*, p21 immunoreactivity was also detected in the nuclei of the endometrial carcinoma cells. *C*, a case of negative immunoreactivity for PPAR γ in the normal proliferative-phase endometrium. *D*, immunoreactivity for PPAR γ was detected in the normal secretory-phase endometrium. *E*, immunoreactivity for PPAR γ was detected in the nuclei of epithelial cells in normal mammary glands. *F*, PPAR γ immunoreactivity was positive in the nuclei of adipocytes.

patients with hypertension was 56 (55.4%) and the number of patients with type II diabetes was 24 (23.8%). A significant association was detected between obesity and disease-free survival ($P = 0.0353$; Fig. 1), but there were no significant correlations between obesity and overall survival of the patients ($P = 0.0844$; data not shown).

Immunohistochemistry and correlation with clinicopathologic variables. Positive immunoreactivity for PPAR γ was detected in the nuclei of carcinoma cells, normal endometrial gland cells, and hyperplastic cells (Fig. 2A and C-E). PPAR γ immunoreactivity was detected in 67 of 103 (65%) of carcinoma cases,

11 of 23 (48%) of proliferative-phase endometrium, 14 of 19 (74%) of secretory-phase endometrium, and 27 of 32 (84%) of endometrial hyperplasia. Immunoreactivity of PPAR γ was significantly lower in endometrial carcinoma than in secretory-phase endometrium ($P < 0.0001$) and endometrial hyperplasia ($P = 0.0015$). Lower immunoreactivity was also detected in proliferative endometrium than in secretory-phase endometrium ($P = 0.0163$) and endometrial hyperplasia ($P = 0.0008$; Fig. 3A). Results of immunohistochemistry for PPAR γ and their correlation with clinicopathologic variables, such as age, stage, and grade of the patients, are summarized in Table 2.