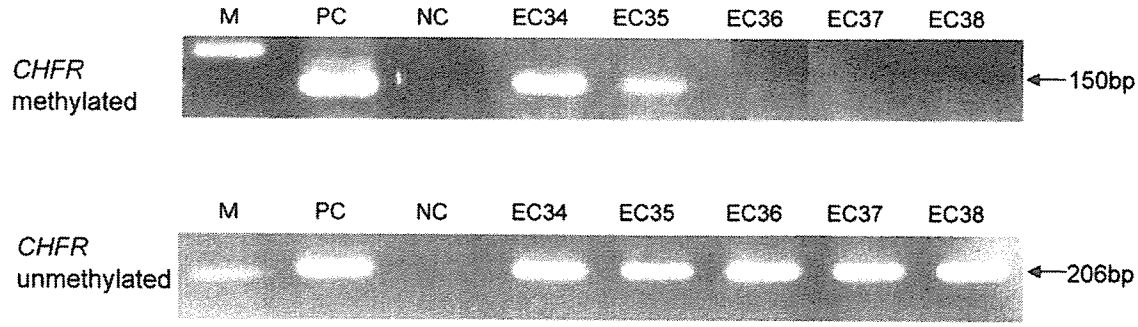

Figure 1. MSP analysis of *CHFR* in endometrial cancer cells obtained using cytology. MSP analysis was conducted with DNA extracted from specimens of endometrial cancer. Bands due to aberrant hypermethylation are found in lanes EC34 and EC35. M, marker; PC, positive control; NC, negative control; EC, endometrial cancer.

to 80% confluence. Paclitaxel (Bristol-Myers Squibb Co., NY, USA) was then added until its final concentration in the culture medium was 1.0 μg/ml. Forty-eight hours later, the cells were treated with trypsin, washed twice with PBS, and then centrifuged at 15,000 rpm for 5 min. The supernatant was removed and the cell pellet was washed with 500 μl of PBS. The vortexed cells were combined with 1 ml of 100% cold ethanol and then incubated at room temperature for 30 min for fixation. After rinsing twice with PBS and removing the supernatant, 500 μl of RNase was added to the cell pellet and the mixture was incubated at room temperature for 20 min. After a further addition of 500 μl of propidium iodide (PI), the cells were strained with a cell strainer and cell cycle status was determined using an EpicsXL MCL flow cytometer (Beckman Coulter Inc., Fullerton, CA, USA).

Anticancer drug-sensitivity test. A collagen gel droplet-embedded culture drug-sensitivity test (CD-DST) was performed using 6 endometrial cancer-derived cell lines and 12 of 50 specimens of endometrial cancer (6). The culture cells and specimens were treated with a cell-dispersing enzyme, EZ (Nitta Gelatin Inc., Tokyo, Japan), for 2 h and centrifuged. The cells were preincubated in a collagen gel flask for 24 h and the living cells that attached to the collagen gel were collected. Cellmatrix Type CD solution was added to these cells, and then 3 drops of a 30-μl/drop collagen gel cell suspension were placed in a 6-well plate. The cell suspension was left to stand for 1 h in an incubator under 5% CO_2 at 37°C, and after gelation the medium was doubled to 4 ml/well and anticancer drugs were added. Four anticancer drugs, cisplatin, doxorubicin, paclitaxel and docetaxel, were used at final concentrations of 2.0, 0.02, 1.0 and 0.1 μg/ml, respectively. Twenty-four hours after drug administration, the cells were washed to remove the anticancer drugs and then incubated without serum for 7 days under 5% CO_2 at 37°C. After staining with Neutral Red, the cells were fixed with formalin and dried, and cell images were processed using an image analyzer. In the CD-DST, drug sensitivity is assessed using the ratio (T/C) of the number of living cells cultured in a solution containing anticancer drug (T) to that of control cells cultured in a solution without anticancer drug (C).

Expression analysis of CHFR protein. Specimens of endometrial cancer (G3) from 4 patients with aberrant hypermethylation of *CHFR* were embedded with OCT compound and frozen in liquid nitrogen. Cryostat-sliced sections were applied to slides and fixed with 100% ethanol, and the slides were incubated at 4°C overnight with primary antibody (anti-*CHFR* antibody; Santa Cruz, Delaware, CA, USA) diluted 100-fold with 1% BSA in PBS. After rinsing three times with PBS, the slides were incubated with secondary antibody (biotin-labeled anti-goat IgG) at room temperature for 30 min, and after three further rinses with PBS the slides were incubated with ABC (avidin-biotin peroxidase) complex at room temperature for 30 min. After further rinsing three times with PBS, the slides were treated with 0.2 mg/ml diaminobenzidine (DAB) for about 5 min as a color reaction. After rinsing twice with PBS, the slides were treated with hematoxylin solution for nuclear staining, then dehydrated and enclosed, and observed microscopically. Immunohistochemical data for staining of *CHFR* protein were assessed using the following criteria: specimens with 30% or more of stained tumor cells were considered positive, and specimens with <30% of tumor cells showing staining were considered negative.

Results

Partial results of MSP analysis of endometrial cancer cells obtained using liquid-based cytology are shown in Fig. 1. Endometrial cancer specimens had a 12.0% (6/50) frequency of aberrant hypermethylation of the promoter region of *CHFR*, whereas specimens of atypical endometrial hyperplasia and normal endometrial cells in the proliferative and secretory phases showed no aberrant hypermethylation of the *CHFR* promoter region (Fig. 1, Tables II and III).

Correlations of aberrant DNA hypermethylation of the *CHFR* promoter with clinicopathological factors were examined in endometrial cancer patients. The frequency of aberrant hypermethylation in G3 adenocarcinoma was significantly higher than in G1 adenocarcinoma ($p<0.05$). Aberrant DNA hypermethylation is also generally thought to increase with age, but no significant difference in mean age was found between patients with and without aberrant hypermethylation of *CHFR*. Therefore, these data do not indicate that aberrant hypermethylation occurs more frequently in elderly patients with endometrial cancer (Table IV).

Of the 6 culture cell lines derived from endometrial cancer, SNG-II and HEC108 cells showed aberrant hypermethylation

Table II. Frequency of aberrant DNA hypermethylation of *CHFR* in specimens of endometrial cancer.

No.	Age	Tissue type	Stage	Differentiation	*CHFR*
EC1	52	EM	Ib	G3	U
EC2	50	EM	Ia	G1	U
EC3	51	EM	IIIc	G1	U
EC4	54	AS	IIIc	G3	M
EC5	51	EM	Ia	G1	U
EC6	61	EM	Ib	G1	U
EC7	70	EM	IIIc	G2	U
EC8	61	EM	IIb	G1	U
EC9	62	AS	IIIa	G2	U
EC10	40	EM	IIa	G1	U
EC11	59	EM	IIa	G3	U
EC12	57	EM	Ib	G3	U
EC13	80	EM	IIIc	G3	U
EC14	54	AS	Ib	G1	U
EC15	53	EM	Ib	G3	U
EC16	42	EM	IIb	G1	U
EC17	71	EM	IIIc	G3	U
EC18	60	EM	Ib	G1	U
EC19	57	EM	IIIa	G2	U
EC20	71	EM	IIa	G1	U
EC21	37	EM	IIa	G2	U
EC22	47	EM	IIIb	G1	U
EC23	67	EM	Ic	G2	M
EC24	53	EM	Ia	G1	U
EC25	69	EM	IIIc	G2	U
EC26	55	EM	IIIc	G2	U
EC27	54	EM	Ia	G1	U
EC28	63	EM	Ia	G1	U
EC29	41	EM	Ib	G1	U
EC30	62	AS	Ib	G1	U
EC31	58	EM	Ib	G2	U
EC32	56	EM	IIIc	G3	M
EC33	71	EM	Ib	G2	U
EC34	53	AS	Ib	G3	M
EC35	50	EM	IIIa	G3	M
EC36	42	AS	IIIc	G3	U
EC37	55	EM	Ic	G3	U
EC38	34	AS	IIIc	G1	U
EC39	61	EM	Ic	G1	U
EC40	61	EM	Ic	G1	U
EC41	61	EM	Ib	G1	U
EC42	59	EM	Ib	G1	U
EC43	55	AS	IVb	G2	U
EC44	54	EM	IIa	G1	U
EC45	78	EM	Ib	G3	U
EC46	65	EM	Ib	G2	M
EC47	68	EM	IIIc	G3	U
EM48	54	EM	IIIc	G2	U
EM49	60	EM	Ib	G1	U
EC50	70	EM	IVb	G2	U

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

Table III. Frequency of aberrant DNA hypermethylation of *CHFR* in cells of normal endometrium and atypical endometrial hyperplasia.

No.	Age	Tissue type	*CHFR*
AE1	30	AEH	U
AE2	32	AEH	U
AE3	35	AEH	U
AE4	35	AEH	U
AE5	46	AEH	U
AE6	41	AEH	U
AE7	50	AEH	U
AE8	45	AEH	U
AE9	47	AEH	U
AE10	45	AEH	U
NE1	51	Sec	U
NE2	52	Sec	U
NE3	44	Sec	U
NE4	23	Sec	U
NE5	34	Sec	U
NE6	43	Pro	U
NE7	42	Pro	U
NE8	44	Pro	U
NE9	32	Pro	U

AE, atypical endometrial hyperplasia; NE, normal endometrium; AEH, atypical endometrial hyperplasia; Sec, secretory phase; Pro, proliferative phase; U, unmethylated.

Table IV. Correlation of aberrant DNA hypermethylation of *CHFR* with histological differentiation, stage at surgery and mean onset age.

	CHFR		
	Methylated	Unmethylated	P-value
G1	0	23	
G2	2	11	<0.05
G3	4	10	
Stage			
I	3	22	
II	0	7	NS
III	3	13	
IV	0	2	
Mean onset age	57.5±6.90	57.4±10.34	NS

G1, well-differentiated; G2, moderately differentiated; G3, poorly differentiated.

of *CHFR*, and RT-PCR analysis of *CHFR* expression showed reduced mRNA levels in these cells (Fig. 2). Consistent with this observation, the SNG-II and HEC108 cells showed higher

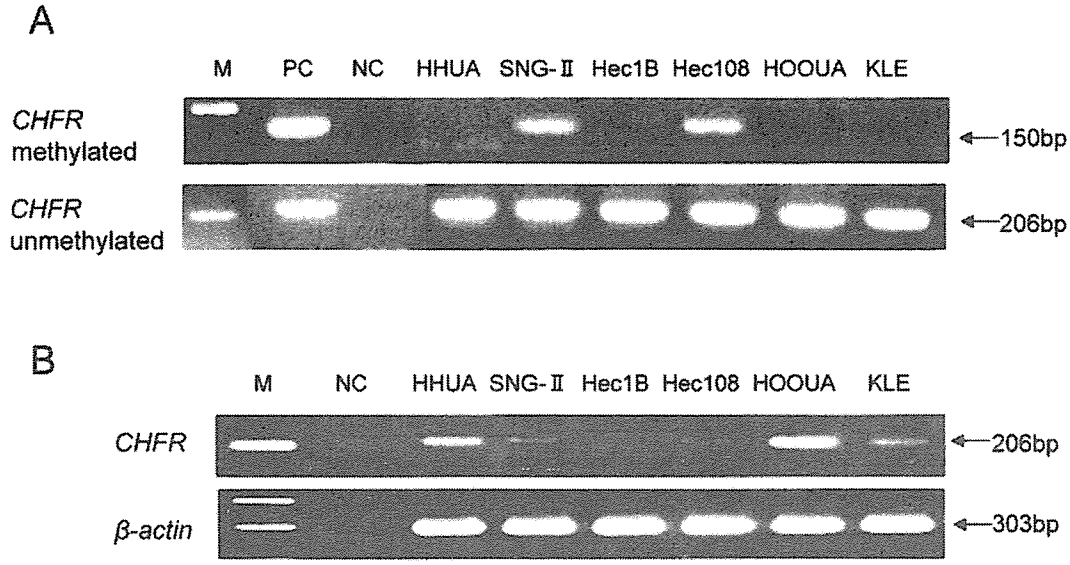

Figure 2. (A), MSP analysis of *CHFR* in endometrial cancer-derived cell lines. Aberrant hypermethylation of *CHFR* was found in two cell lines, SNG-II and HEC108. (B), Expression analysis of CHFR in endometrial cancer-derived cell lines using RT-PCR. *CHFR* expression was reduced in SNG-II and HEC108 cells, which showed aberrant hypermethylation of CHFR. M, marker; PC, positive control; NC, negative control.

Table V. Sensitivity (T/C ratio) of endometrial cancer-drived cells to various anticancer drugs in the CD-DST.

Cell line	*CHFR*	Cisplatin (%)	Doxorubicin (%)	Paclitaxel (%)	Docetaxel (%)
HHUA	U	100	100	22.5	31.0
SNG-II	M	52.0	65.1	16.7	15.1
Hec1B	U	91.1	85.7	71.2	65.9
Hec108	M	56.6	76.6	11.8	20.6
HOOUA	U	94.2	88.4	20.7	50.9
KLE	U	57.6	93.6	60.8	63.9

M, methylated; U, unmethylated.

sensitivity to paclitaxel and docetaxel in the CD-DST, compared to that of other cell lines (Table V).

Following treatment of SNG-II cells with 5-aza-dC, the aberrant hypermethylation band in the MSP analysis was weaker than that before administration of 5-aza-dC, and recovery of *CHFR* expression was shown in these cells (Fig. 3). Differences in sensitivity of SNG-II and HEC108 cells, and of KLE cells (which do not show aberrant hypermethylation of *CHFR*), to four anticancer drugs were examined using the CD-DST before and after 5-aza-dC administration. The T/C ratios of cells treated with cisplatin and doxorubicin did not differ before and after 5-aza-dC administration, regardless of the presence or absence of aberrant hypermethylation of *CHFR*, showing that 5-aza-dC administration had no effect on sensitivity to cisplatin and doxorubicin. Similarly, the T/C ratios of KLE cells treated with paclitaxel and docetaxel were unchanged by 5-aza-dC administration. However, the T/C ratios in SNG-II and HEC108 cells treated with paclitaxel and docetaxel significantly increased after 5-aza-dC administration, showing that these cells initially had low sensitivity to taxanes (Table VI).

Cell cycle changes in SNG-II and KLE cells treated with paclitaxel alone or combined paclitaxel and 5-aza-dC were determined using flow cytometry. The percentages of paclitaxel-treated KLE cells in the G2/M and Sub-G1 phases were 67.3 and 5.1%, respectively; these data were almost the same as those for untreated control cells. KLE cells treated with paclitaxel and 5-aza-dC gave similar results. In contrast, the percentage of paclitaxel-treated SNG-II cells in the G2/M phase was very low (0.2%) and the percentage of these cells in the Sub-G1 phase was higher (13.3%) compared to control cells, indicating that paclitaxel administration induced apoptosis. However, with combined paclitaxel and 5-aza-dC treatment, the percentage of SNG-II cells in the G2/M phase was high (82.7%) and that for cells in the Sub-G1 phase was low (1.8%) compared to control cells; a similar pattern to that seen for paclitaxel-treated KLE cells (Fig. 4).

MSP analysis indicated aberrant hypermethylation of *CHFR* in 12.0% (6/50) of endometrial cancer specimens, with this being particularly common for G3 specimens (4/14, 28.6%). Immunohistochemical analysis was conducted on specimens showing aberrant hypermethylation of *CHFR* from

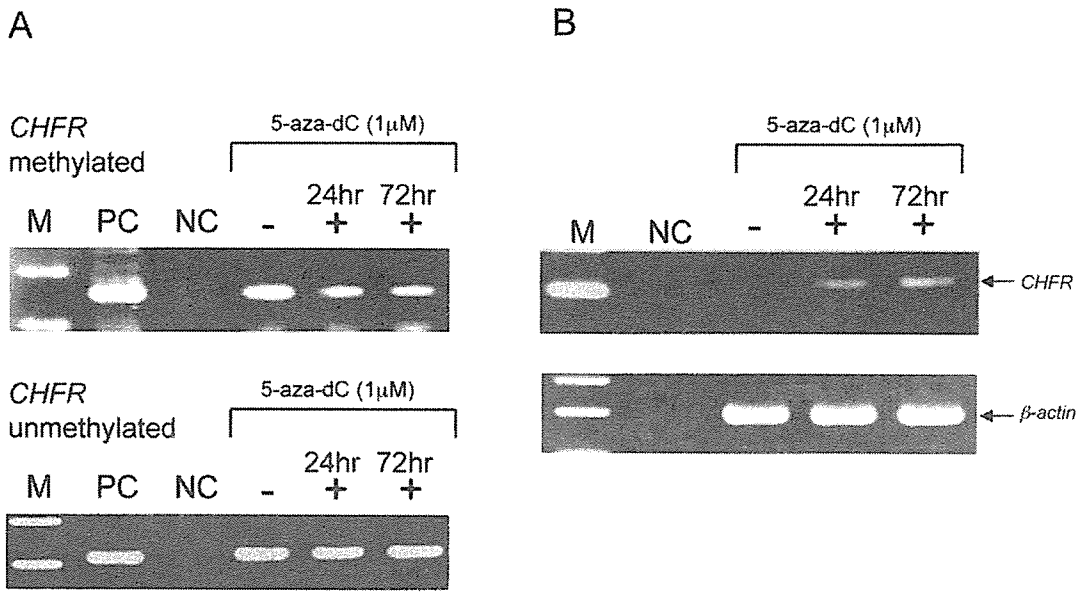

Figure 3. Demethylation analysis of *CHFR* in SNG-II cells. (A), In MSP, bands due to aberrant hypermethylation reduced with time after 5-aza-dC administration. (B), RT-PCR analysis showed recovered *CHFR* expression 24 h after the second 5-aza-dC administration. M, marker; PC, positive control; NC,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

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

		Cisplatin (%)		Doxorubicin (%)		Paclitaxel (%)		Docetaxel (%)	
Cell line	*CHFR*	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

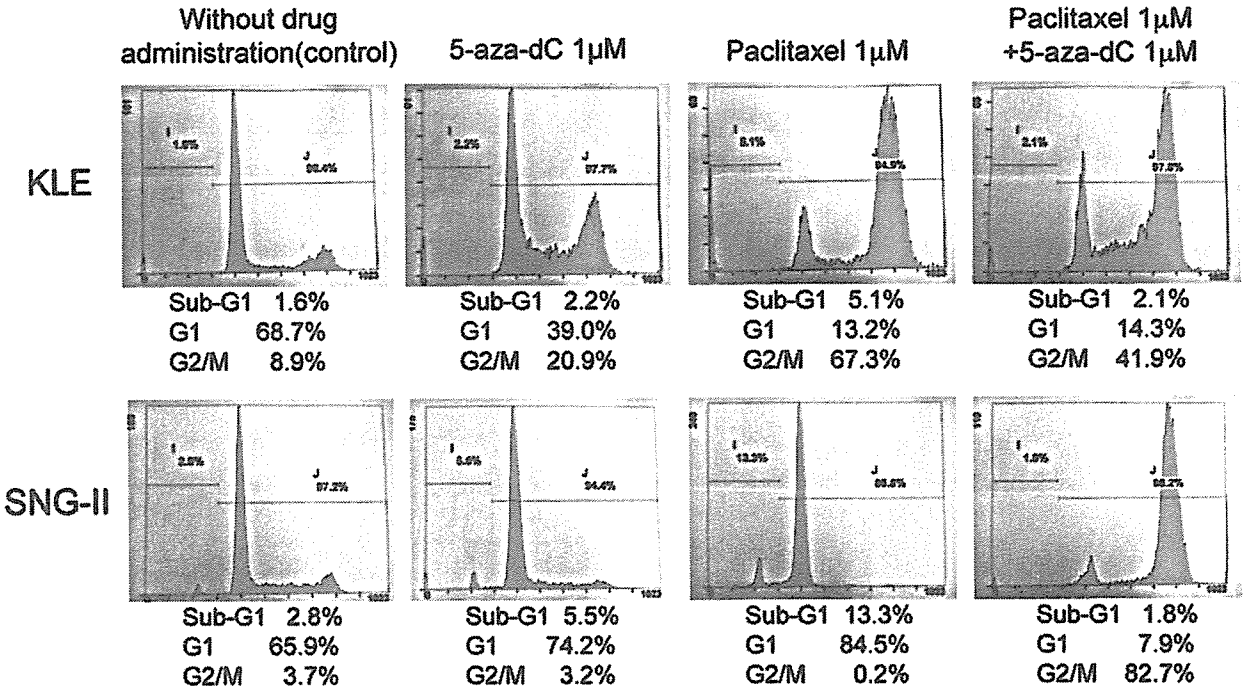

Figure 4. Cell cycle analysis in KLE and SNG-II cells using flow cytometry. KLE cells treated with paclitaxel alone and with combined paclitaxel and 5-aza-dC showed cell accumulation in G2/M phase and no significant change in the number of cells in Sub-G1 phase. SNG-II cells treated with paclitaxel alone 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.

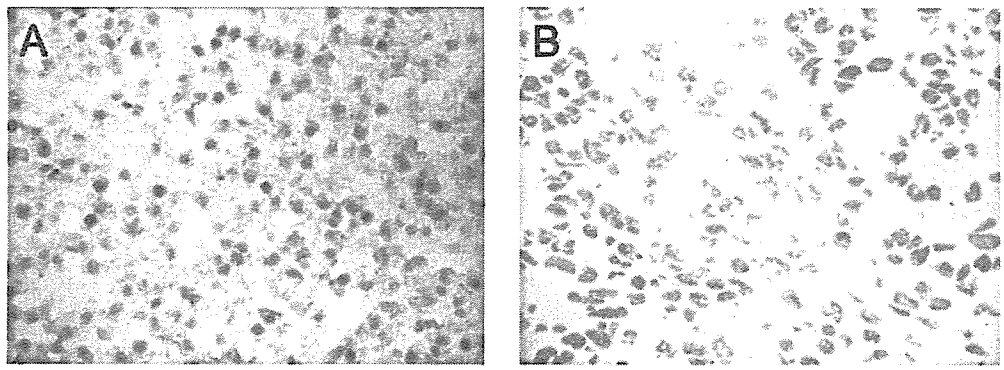

Figure 5. Immunohistochemical analysis of CHFR protein expression in patients with endometrioid adenocarcinoma (G3) with aberrant hypermethylation of *CHFR*. (A), EC35 staining; (B), EC32 staining. The nuclei of one of the four G3 adenocarcinoma patients stained positively (A), but three patients had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

No.	Tissue type	Stage	Differentiation	*CHFR*	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☆

Yukiharu Todo [a,*], Kazuhira Okamoto [b], Masaru Hayashi [c], Shinichiro Minobe [d], Eiji Nomura [e], Hitoshi Hareyama [f], Mahito Takeda [a], Yasuhiko Ebina [a], Hidemichi Watari [a], Noriaki Sakuragi [a]

[a] *Department of Obstetrics and Gynecology, Hokkaido University School of Medicine, Kita-15, Nishi-7, Kita-Ku, Sapporo 060-8638, Japan*
[b] *Division of Obstetrics and Gynecology, Asahikawa Kosei General Hospital, Asahikawa, Japan*
[c] *Division of Obstetrics and Gynecology, Obihiro Kosei General Hospital, Obihiro, Japan*
[d] *Division of Obstetrics and Gynecology, Kushiro Red Cross Hospital, Kushiro, Japan*
[e] *Division of Obstetrics and Gynecology, Oji General Hospital, Tomakomai, Japan*
[f] *Division of Obstetrics and Gynecology, Sapporo City General Hospital, Sapporo, Japan*

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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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* Corresponding author. Fax: +81 11 706 7711.
E-mail address: yukiharu@med.hokudai.ac.jp (Y. Todo).

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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 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.

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.

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%)

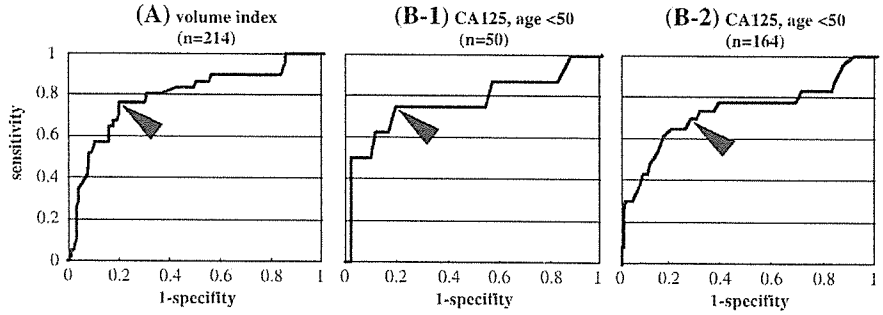

Fig. 1. (A) Receiver operating characteristic curve obtained from the relationships between volume index and LNM in the pilot study. Cut-off value (arrow) for LNM was 36. (B) Receiver operating characteristic curves obtained from the relationships between serum CA125 level and LNM. Cut-off value (arrow) 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	Multivariate analysis			
	n/N	%	*p*-value	*β*	SE	OR (95% CI)	*p*-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)				
Ia	21	0	0	0
Ib	91	11	10	10
Ic	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% (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.

Table 5
LNM frequencies according to LNM score

LNM score		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).

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

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

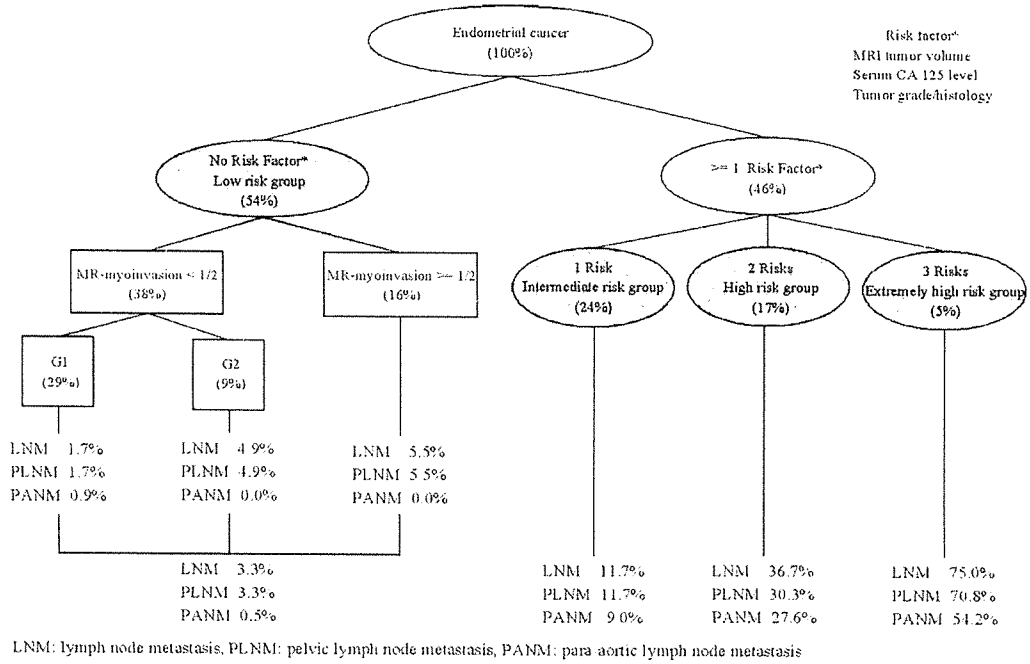

Fig. 2. Rate of LNM for the combined cohorts of 425 patients with endometrial carcinoma according to LNM score and distribution map of patients estimated from a previous paper [4].

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

Sumika Saito,[1] Kiyoshi Ito,[1,3] Satoru Nagase,[1] Takashi Suzuki,[2] Jun-Ichi Akahira,[2] Kunihiro Okamura,[1] Nobuo Yaegashi[1] and Hironobu Sasano[2]

Departments of [1]Obstetrics and Gynecology and [2]Pathology, Tohoku University Graduate School of Medicine, Sendai 980-8574, Japan

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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 NH_2 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 promotor-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.*

[3]To whom correspondence should be addressed.
E-mail: kito@mail.tains.tohoku.ac.jp

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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-oopholectomy, 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-oopholectomy 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/m^2 cisplatin, 40 mg/m^2 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 ERα, ERβ 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; ERα, 1/50; ERβ, 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% H_2O_2), 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 ERα and ERβ. 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, ERα, ERβ and Ki-67 was carried out in high-power fields (×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 $MgCl_2$, 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

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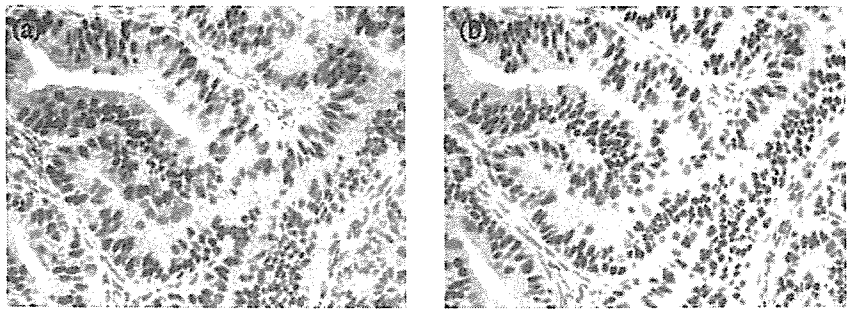

Fig. 1. Immunohistochemical staining for (a) progesterone receptor A (PRA) and (b) progesterone receptor B (PRB) in endometrioid endometrial carcinoma. PRA and PRB immunoreactive proteins were detected in the nuclei of carcinoma cells of G1 adenocarcinoma. Original magnification, ×400.

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 Light Cycler System (Roche Diagnostics, Mannheim, Germany) using the DNA-binding dye SYBER Green I (Roche Diagnostics). The 20-μL reaction mixture contained 3 mM $MgCl_2$ 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, ACACCTTGCCTGAAGTTTCG and PRB 3′ antisense, CTGTCCTTTTCTGGGGGACT (196 bp); β-actin 5′ sense, CCAACCGCGAGAAGATGAC and β-actin 3′ antisense, GGAAGGAAGGCTGGAAGAGT (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, ERα LI, ERβ 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). ERα, ERβ 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 ERα 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;

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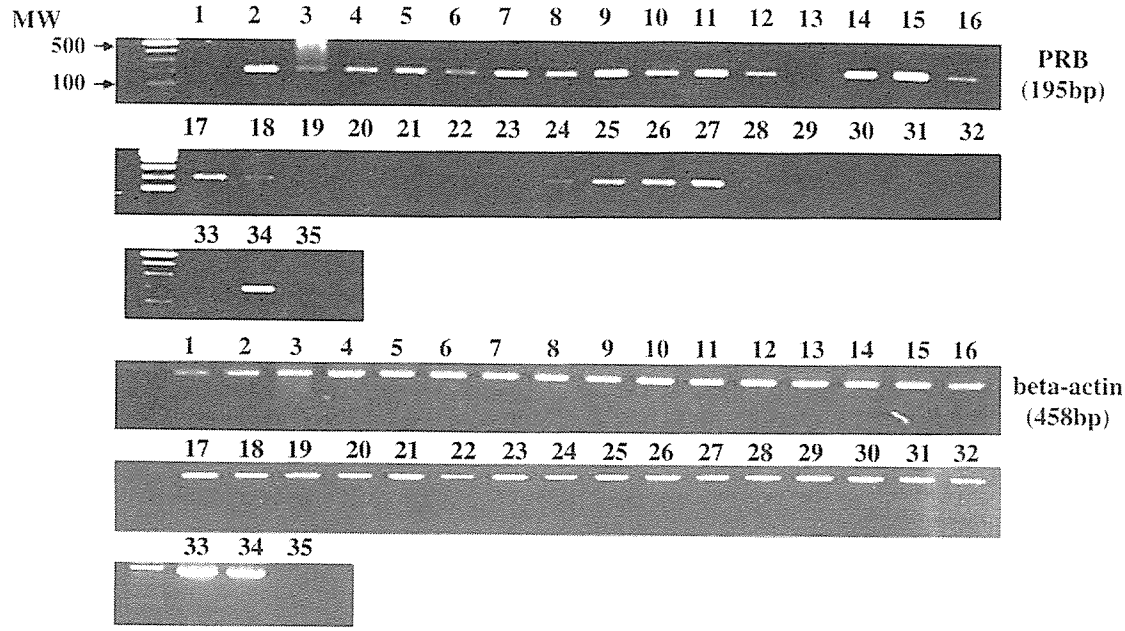

Fig. 2. Reverse transcription–polymerase chain reaction (RT-PCR) analysis of total RNA extracted from endometrioid endometrial carcinoma. Nos 4, 10, 13, 21, 29, 30, 31, 32 and 33 are progesterone receptor B (PRB)-negative cases, as determined by immunohistochemistry. No. 34 is a positive control. No. 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).

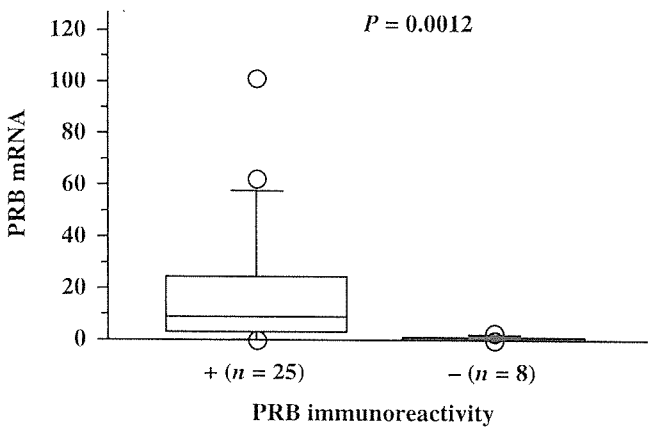

Fig. 3. Correlation between progesterone receptor B (PRB) immunoreactivity and its mRNA level determined by quantitative reverse transcriptin–polymerase chain reaction analyses in human endometrial carcinoma. There was a statistically significant positive correlation between PRB immunoreactivity and 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

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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			PRB		
		+ (n = 51)	− (n = 52)	P-value	+ (n = 79)	− (n = 24)	P-value
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
ERα LI (median)	23	34	11	**0.003**	34	4.5	**<0.0001**
ERβ 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 (≤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**
ERα (positive vs negative)	**0.0426**	0.2667
ERβ (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

Preditor	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
ERα (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**
ERα (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.

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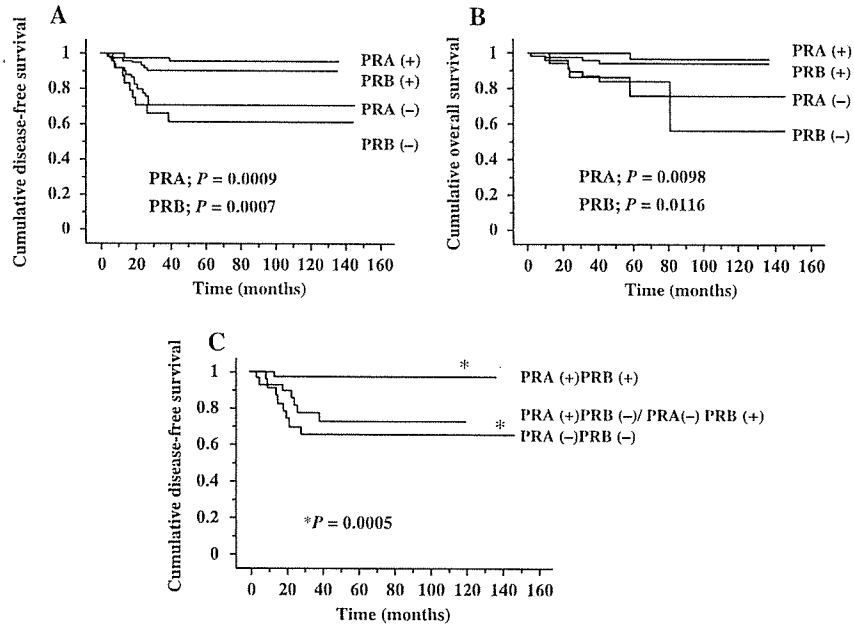

Fig. 4. Correlation between progesterone receptor A (PRA) or B (PRB) isoform immunoreactivity and (A) recurrence, and (B) survival for patients with endometrioid endometrial carcinoma. (C) Correlation between immunoreactivity for both isoforms and recurrence for patients with endometrioid endometrial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

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熱可逆性ハイドロゲル (Themoreversible gelation polymer; TGP) を培養基材に用いた再発した婦人科癌に対する抗癌剤感受性試験

大原　樹[1]　木口　一成[1]　和田　康菜[1]　鈴木　直[1]
小林　陽一[1]　月川　賢[2]　石塚　文平[1]

（受付：平成18年8月17日）

抄　録

熱可逆性ハイドロゲル (Themoreversible gelation polymer; TGP) は，転移温度(22°C)の上下でゾル・ゲル状態が可逆的に変化する高分子化合物である。TGP 内で固形癌組織は三次元的に増殖するのに対して，繊維芽細胞は増殖を認めない特徴を有する。再発した婦人科癌に対してより有効な化学療法を計画するため，この新しい培養用基材を用いて抗癌剤感受性試験を施行し臨床応用することを目的とする。再発した婦人科癌患者45例(子宮頸癌7例，子宮体癌5例，卵巣癌33例)を対象患者とし，十分なインフォームドコンセントのもと，再発腫瘍の生検あるいは手術摘出を行った。手術より切除された検体(癌組織)を0.5 mm 厚に細切した。24 well プレートに冷却によってゾル状態となった TGP を注入し，37°C で4日間培養した。培養した組織に対し TGP を用いた感受性試験を施行し，CDDP (cisplatin), MMC (mitomycin C), 5FU (5-fluorouracil), PTX (paclitaxel), CPT-11 (irinotecan) に関する IC_{50} (inhibition concentration; 50% 抑制濃度) 値を求めた。45例中，IC_{50}値を算出できた41例(91.1%)において判定可能であった。個々の症例に応じて，感受性を認めた抗癌剤を用いオーダーメードな化学療法(点滴静注，動注療法など)を施行した。評価可能病変を有した症例の奏効率は子宮頸癌66.7%，子宮体癌33.3%，卵巣癌42.3%，全体45.7% であった。また，真陽性率45.7%，真陰性率100%，感度100%，特異度9.5% であり臨床効果予測率は48.6% であった。新しい培養用基材 (TGP) を使用した感受性試験は，再発した婦人科癌患者の化学療法の個別化を目指すうえで有効な手段であると考えられる。

索引用語

抗癌剤感受性試験，熱可逆性ハイドロゲル (Themoreversible gelation polymer; TGP)，組織培養，婦人科癌

緒　言

近年わが国において，婦人科癌に対する多剤併用化学療法の投与計画は多岐にわたっており，初回治療は臓器別，組織別に選択される傾向にあり，再発・再燃時には十分なエビデンスを伴わない初回治療と異なった投与計画が試行されているに過ぎない。また，化学療法後に短期間で再発した癌に対しては，抗癌剤の感受性が低く，有効な化学療法が確立されていない[1]。このような再発患者は前治療による毒性のため，強力な化学療法が行えないことも

1 聖マリアンナ医科大学　産婦人科
2　同　外科学(消化器・一般外科)