

Determining Best Potential Predictor during High-dose Progestin Therapy for Early Staged and Well-differentiated Endometrial Adenocarcinoma Using Semiquantitative Analysis Based on Image Processing and Immunohistochemistry

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Abstract

This study aimed to examine whether morphological changes during the early stage of treatment or indices of proliferation, apoptosis, or hormone receptors are reliable predictors of the hormonal response to uterus-preserving high-dose progestin therapy in patients with endometrial adenocarcinoma. Seven patients (5 good responders and 2 poor responders) with presumptive stage IA endometrial adenocarcinoma treated with 600 mg/day of medroxyprogesterone acetate were reviewed. Epithelial cell size and stromal area observable on microscopic examination of hematoxylin and eosin-stained sections, and immunostaining labeling indices for Ki-67 nuclear antigen, single-stranded DNA, estrogen receptor, and progesterone receptor were semiquantitatively analyzed before treatment and after 4, 8, 12, and 16 weeks of treatment using computer imaging programs. The mean ratio of cell size after 4 weeks of treatment to that before treatment in good responders was 3.83, whereas the ratios in the 2 poor responders were 1.08 and 0.98. The mean Ki-67 nuclear antigen labeling index before treatment was 37.2% for the 5 good responders but was 51.0% in the 2 poor responders. The indices of the poor responders remained high (20%–77%), even after 16 weeks of treatment; in contrast, the indices of the good responders were low (0.4%–7.3%) throughout the treatment period. No definitive differences in labeling indices for single-stranded DNA, estrogen receptor, or progesterone receptor were observed between good and poor responders or at different stages of treatment ($p>0.05$). In conclusion, a higher epithelial cell size ratio after 4 weeks of treatment in conjunction with lower Ki-67 nuclear antigen labeling indices could be a potential predictor of hormonal response.

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Key words: high-dose progestin therapy, immunohistochemistry, medroxyprogesterone acetate, semiquantitative analysis, uterine preservation

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Introduction

Although conservative therapy with a high-dose progestin is an innovative treatment for endometrial adenocarcinoma in young patients who wish to remain fertile^{1,2}, the treatment poses great risks because of the patients' young age. At the start of treatment, the disease must be presumptively staged on the basis of the results of diagnostic imaging examinations, such as magnetic resonance imaging (MRI) and computed tomography (CT), not on the basis of surgery, and the histological diagnosis must be based on blind methods, such as endometrial curettage, not on the thorough examination of resected specimens.

Fujiwara et al. have reported an unsuspected case of uterine carcinosarcoma (heterologous) diagnosed following conservative therapy with medroxyprogesterone acetate (MPA) for presumed early-stage endometrial carcinoma³. Among the cases that we have treated with high-dose MPA, one case was not initially recognized as a serous carcinoma because of the small foci. Therefore, a reliable predictor of responsiveness to hormonal therapy is needed so that treatment for hormone-unresponsive cases can be stopped early and hysterectomy can be performed.

We recently have reported that responsive cases show homogeneous histologic change during high-dose progestin therapy with MPA until cure⁴. Swelling of carcinomatous epithelial cells, a decrease in mitotic activity, and an increase in stromal area were observed in all such cases. However, in contrast to typical cases, the case of serous carcinoma mentioned above gradually showed malignant features. Thus, the main purpose of the present study was to assess histological characteristics during therapy for their suitability as predictors of hormonal response using semiquantitative analysis based on the results of image processing and immunohistochemical studies.

Materials and Methods

Patients

Of the patients who visited the Department of Obstetrics and Gynecology, Nippon Medical School Chiba Hokusoh Hospital, from 1997 through 2006, 30 patients received a presumptive diagnosis of stage Ia endometrial adenocarcinoma of grade 1 endometrioid type according to the classification of the International Federation of Gynecology and Obstetrics (FIGO). Nine of these patients started to receive a high-dose progestin (MPA, 600 mg per day) as conservative therapy for uterine preservation according to the treatment regimen described in **Table 1**. However, upon subsequent review of the initial diagnostic specimens, 2 of the 9 patients were found to have atypical complex endometrial hyperplasia alone, and, thus, the 7 remaining patients were presumed to have endometrial adenocarcinoma of pure grade 1 endometrioid histologic type at diagnosis and were enrolled in this study.

During treatment (as described in **Table 1**), complete disappearance of the carcinoma foci was observed histologically within 12 weeks in 1 patient and within 16 weeks in 4 patients, whereas residual foci remained even after 16 weeks in the remaining 2 patients. The former 5 patients were classified as good responders and, as such, daily administration of 200 mg of MPA was subsequently continued for an additional 4 months, followed by daily administration of 10 mg of MPA to maintain conservative therapy. The latter 2 patients, who were classified as poor responders, subsequently underwent hysterectomy. Therefore, another purpose of this study was to compare differences in the histological characteristics between the 2 patients who were poor responders and the 5 patients who were good responders.

This study was approved by the Institutional Review Board of Nippon Medical School Chiba Hokusoh Hospital, and informed consent was obtained from all patients after sufficient explanation had been provided.

Table 1 Treatment regimen of high-dose progestin therapy for uterine preservation at the Nippon Medical School Chiba Hokusoh Hospital

For nulliparous women hoping to preserve the uterus
<u>MRI, CT, and curettage at week 0</u>
<i>If presumptive FIGO stage Ia and grade 1 endometrioid adenocarcinoma</i>
- No myometrial invasion on T2-weighted pelvic MRI
- No swelling of pelvic and paraaortic lymph nodes on CT
- No space-occupying lesion on CT
- Grade 1 endometrioid adenocarcinoma on H&E-stained slides
→ Start MPA* at 600 mg/day
<i>Otherwise</i>
→ Surgical treatment including hysterectomy
<u>Curettage after 4, 8, and 12 weeks of treatment</u>
<i>If worse histologic findings worse than before</i>
→ Surgical treatment including hysterectomy
<i>Otherwise</i>
→ Continue MPA at 600 mg/day
<u>Curettage at 16 weeks</u>
<i>If any residual carcinomatous focus</i>
→ Surgical treatment including hysterectomy
<i>Otherwise</i>
→ Continue MPA at 200 mg/day for an additional 4 months then MPA at 10 mg/day until expecting pregnancy

*MPA-medroxyprogesterone acetate

Biopsy Specimen Preparation

Each endometrial curettage specimen was fixed in 10% buffered formalin, embedded in paraffin, and cut into 4- μ m sections. The sections were routinely stained with hematoxylin and eosin (H&E) and immunohistochemical agents. Immunohistochemical staining was performed with the avidin-biotin-peroxidase-complex technique using a Dako LSAB kit (Dako, Carpinteria, CA, USA) and primary antibodies against Ki-67 nuclear antigen (Immunotech [Cedex, France]; MIB-1, 1 : 50 dilution), single-stranded DNA (ssDNA) (Dako; polyclonal, 1 : 400 dilution), estrogen receptor (ER) (Immunotech; ER1D5; 1 : 50 dilution), and progesterone receptor (PR) (Immunotech; PR10A9; 1 : 50 dilution).

For antigen retrieval, specimens in 10 mM sodium citrate buffer (pH 6.0) were pretreated in a microwave oven at 95°C for 30 minutes for MIB-1, ER, and PR, and specimens were also pretreated with proteinase K for ssDNA. As positive controls, sections from tonsil tissue with follicular hyperplasia were used for MIB-1 and ssDNA, and those from ductal breast carcinoma were used for ER and PR. Negative control sections were prepared using

nonimmune γ -globulin in place of the primary antibody. All sections were counterstained with H&E and mounted in glycerin jelly.

Diagnosis

Endometrial adenocarcinoma was diagnosed according to the criteria of Silverberg and Kurman⁵. In all patients, atypical glands proliferated in a compact fashion with a “back-to-back” appearance, presenting stronger nuclear atypia with large, round nuclei and a vesicular chromatin pattern, obvious stromal invasion with no stromal interposition, and poorly defined intercytoplasmic borders.

Study Design and Analysis

According to our previous report, the first change observed in all good responders to high-dose progestin therapy is swelling of the neoplastic epithelial cells with mitotic arrest and an increase in stromal area⁴. To determine whether these characteristics could be used as predictors of hormonal response, the following factors were semiquantitatively analyzed in good responders with reference to the 2 poor responders: stroma/tumor

area ratio, change in cellular size, and labeling indices of Ki-67 nuclear antigen, ssDNA, ER, and PR.

Digital microscopic images ($2,074 \times 2,776$ pixels) were obtained with a light microscope (Olympus Vanox AHBS-3; Olympus Optical, Tokyo, Japan) connected to a high-resolution digital camera system (Pixera Pro 600 ES/In studio Ver.1.01; Pixera, San Jose, CA, USA) for each H&E-stained and immunostained biopsy specimen slide prepared at the time of diagnosis (pretreatment) and after 4, 8, 12, and 16 weeks of treatment (posttreatment).

Color painting of specific foci was performed with an imaging software program (Adobe Photoshop 5.0, Adobe Systems Inc., San Jose, CA, USA), and painted areas were measured with a free public-domain software program (I2A Version 1.0; <http://www.studio-fe.hiroishi.org/>). Immunopositive cells and immunonegative cells were marked with black spots and pink spots, respectively, and were automatically counted with an imaging software program (Scion Image Beta 4.0.2, Scion Corporation, Frederick, MD, USA; <http://www.scioncorp.com>).

a) Stroma/tumor area ratio

Digital images of 3 to 5 representative fields at each stage of treatment were obtained for all 7 cases using a $10\times$ antiobjective lens for each H&E-stained slide (**Figs. 1a–g**). First, we colored the stromal portion of each image blue using Adobe Photoshop 5.0 (**Fig. 1h**). Next, we measured the blue area and the total image area in pixels using I2A so that the proportion of the tumor area occupied by stroma could be calculated.

b) Change in cellular size

For all 7 cases, digital images at each stage of treatment were captured with a $40\times$ antiobjective lens for each H&E-stained slide prepared. First, we selected 3 to 5 sites of representative epithelial cells (usually 10 neighboring cells) in each image and then colored them blue using Adobe Photoshop 5.0, after which the blue area was measured in pixels using I2A (**Fig. 1i**). Finally, we calculated the mean size of the epithelial cells and compared each result between pretreatment and posttreatment observations for good responders and poor responders.

Our previous report found that malignant

epithelial cells might become swollen after 4 weeks of treatment. To confirm this observation semiquantitatively, we calculated the swelling ratio, defined as the epithelial cell size ratio at 4 weeks posttreatment/pretreatment. Moreover, we compared swelling ratios between good responders and poor responders to examine differences between them.

c) Labeling indices for Ki-67 nuclear antigen, ssDNA, ER, and PR

Three to 5 digital images were first obtained of each immunostained slide for each antibody. A black spot was marked on each immunopositive cell, and a pink spot was marked on each immunonegative cell in each image (**Fig. 1j**). When these RGB images are converted to gray-scale images, both pink and black markings appear black. However, if only the R component of the RGB image is converted to gray-scale, only black marks appear, and pink marks disappear. Immunopositive cells and immunonegative cells could then be counted by totaling all the above black spots with the Scion Image Beta program, as shown in **Figures 1k and 1l**.

To effectively perform these analyses, the sizes of pink and black marks in Adobe Photoshop had to be decided, and minimal particle size needed to be adjusted in the Scion Image Beta commands regarding the size balance of the nucleus or cytoplasm.

Finally the labeling index for each antibody could be calculated on the basis of the number of positive cells as a percentage of the total number of both positive cells and negative cells, and the resulting indices obtained during the pretreatment to posttreatment periods were compared between good responders and poor responders.

Interobserver Errors

The original diagnostic specimens were reviewed by 3 observers (S.K., O.M., and Y.O.), all of whom are members of the staff of the Nippon Medical School Chiba Hokusoh Hospital. Upon independent review, there were 5 cases in which the diagnoses were concordant among all 3 observers and 2 cases in which the diagnosis was not concordant. In all cases

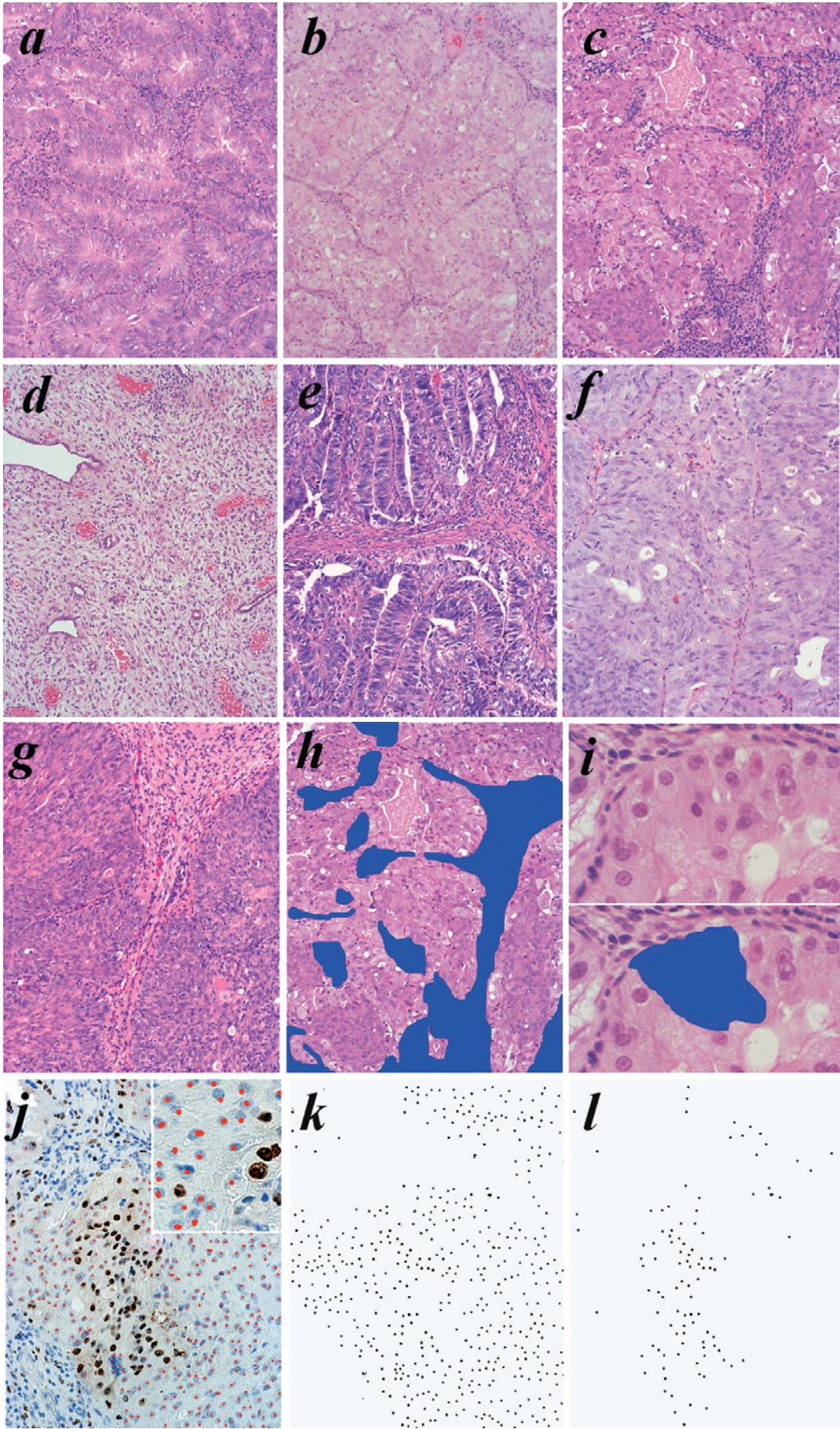


Fig. 1 Semiquantitative analysis through H&E staining and immunohistochemical staining (a–d: serial change in a typical good responder; e–g: serial change in a poor responder; f: estimation of stromal area; i: estimation of cell size; j–l: estimation of immunohistochemistry labeling index.)

- a. A biopsy before treatment shows typical histologic features of grade 1 endometrioid adenocarcinoma: atypical glands proliferating in a compact fashion and several foci showing no stromal interposition and indefinite intercellular borders (patient 1: low-power magnification).
- b. After 4 weeks of MPA treatment, epithelial cells composing the glands show marked swelling and occupy the entire intraglandular lumen (patient 1: low-power magnification).
- c. After 8 weeks of treatment, glands composed of pale swollen epithelial cells are fragmented and surrounded by an inflammatory cell-rich stroma, and a squamous morule appearing adjacent to these swollen epithelial cells (patient 1: low-power magnification).
- d. After 16 weeks of MPA treatment, a biopsy of patient 4 shows atrophic glands and dilated venules dispersed in predecidual stroma. Inflammatory cells are less obvious than after 12 weeks of treatment (patient 1: low-power magnification).
- e. Because the biopsy before treatment in patient 6 showed a crowded assembly of endometrioid-like glands, well-differentiated adenocarcinoma was initially diagnosed, even though the nucleus of the glandular epithelium was larger and more angular than in typical endometrioid tissue (patient 6: low-power magnification).
- f. After 4 weeks of MPA treatment, the cytoplasm appeared hazy and more swollen, and the nucleus was rounder with a homogeneous chromatin character (patient 6: low-power magnification).
- g. After 16 weeks of MPA treatment, malignant epithelial cells with strongly atypical nuclei developed in a more solid pattern than a glandular pattern, and all histologic features had changed completely from those before treatment (patient 6: low-power magnification).
- h. Stromal area of the image shown in Figure 1-c was colored blue with Adobe Photoshop. Total image size was measured to be 5,757,424 pixels, while stroma size was measured to be 1,005,387 pixels with program I2A (patient 1).
- i. Glandular epithelium was swollen after 4 weeks of treatment (upper). The neighboring 10 cells of the image were colored blue for area estimation using Adobe Photoshop (lower) (patient 1).
- j. PR-positive cells were marked with pink dots and PR-negative cells were marked with black dots in immunostained slides after 8 weeks of treatment. A pink dot was put on the nucleus of each immunonegative epithelial cell, and a black dot was put on immunopositive epithelial cells. Inset shows enlarged size (patient 1).
- k. Because gray-scale imaging of the RGB picture displays both black and pink dot marks as black, the number of whole epithelial cells (immunopositive cells and immunonegative cells) was found to be 504 with the Scion Image Beta program (patient 1).
- l. The gray-scale imaging of only the R channel image of the RGB picture could display black dot marks as black, and the number of PR-positive cells was found to be 104 with the Scion Image Beta program. The PR labeling index of the image was therefore calculated to be 21% (patient 1).

the final consensus was mutually agreed upon by all 3 observers. Moreover, the same specimen slides were independently reviewed by an outside observer (M.F.) at another institution (The Jikei University Daisan Hospital) to confirm the sites of stromal invasion.

Statistical Analysis

The differences in the means of each variable between good responders and poor responders during each treatment period were assessed with the Mann-Whitney U-Test by means of a statistical software program (SPSS, SPSS Inc., Chicago, IL, USA).

Results

1. Clinical Features

The clinical features are summarized in **Table 2**. The means of patients' ages, heights, and weights were 29.6 years, 160.7 cm, and 86.6 kg, respectively. The mean body mass index was 33.8 kg/m². Patients did not have any synchronous ovarian cancer or hereditary nonpolyposis colon cancer.

Comorbid conditions included hypertension in 3 of 7 patients (patients 4, 5, and 7), but no patients had diabetes mellitus or polycystic ovary syndrome. Of note, diabetes mellitus developed 4 years later in patient 4, and 5 years later in patient 7. None of the

patients had ever been pregnant. Abnormal vaginal bleeding lasting more than 2 months (patients 1, 2, 4, and 6) was the most common presenting symptom, followed by heavy menstruation (patients 5 and 7) and amenorrhea for more than 2 months (patient 3). All patients had an irregular menstrual history.

The serum CA 125 levels ranged from 6 to 25.7 IU/mL (normal range: <37 mIU/mL; **Table 2**). Histological examination indicated complete disappearance of carcinoma foci within 12 weeks of treatment in 1 good responder (patient 3) and within 16 weeks of treatment in the other 4 good responders (patients 1, 2, 4, and 5). Two good responders (patients 1 and 3), in whom uterine functions were preserved, subsequently married, and 1 patient (patient 1) gave birth. In contrast, the 2 poor responders (patients 6 and 7), who showed residual carcinomatous foci after 16 weeks of treatment, discontinued hormone therapy and underwent hysterectomy.

During treatment, 3 of the 7 patients had occasional nausea, and 2 of these patients complained of mild fatigue. However, none had severe complications, such as deep vein thrombosis and pulmonary arterial embolism. All patients were alive and well at the last follow-up in June 2010, with a mean follow-up period of 8 years 2 months and a median follow-up period of 7 years 3 months.

2. H&E Findings at Diagnosis of Endometrioid Adenocarcinoma

In 6 of the 7 patients, atypical glands proliferated in a compact fashion and had a “back-to-back” appearance in some areas. In all 7 patients, some microscopic fields showed a state of atypical complex endometrial hyperplasia, but most foci showed the more typical findings of adenocarcinoma presenting stronger nuclear atypia with large, round nuclei and vesicular chromatin pattern, obvious stromal invasion with no stromal interposition, and poorly defined intercytoplasmic borders.

In 1 patient, squamous morular metaplasia was present in approximately one-third of the microscopic fields. In another patient, secretory endometrioid adenocarcinoma coexisted with the above-mentioned typical endometrioid

adenocarcinoma. In this patient, the initial histologic diagnosis was presumed to be villoglandular endometrioid adenocarcinoma; however, small foci of serous carcinoma were recognized during review of the diagnostic slides.

3. Candidates for Predictors of Hormonal Response

a) Stroma/tumor area ratio

The proportion of the total tumor area occupied by stroma was, in either good responders (patients 1 through 5) or poor responders (patients 6 and 7), less than 6% at the time of diagnosis and increased in all patients from 10% to 52% after 4 weeks of treatment (**Table 3a**). With further treatment, the stroma of good responders came to occupy more than 95% of the whole tumor area within 12 to 16 weeks, whereas the stroma in each poor responder occupied only 30% or 62% of the whole tumor area, even at 16 weeks. Therefore, the stroma/tumor area ratio became significantly different between good responders and poor responders after 12 weeks of hormonal treatment ($p=0.048$ at 12 weeks, $p=0.067$ at 16 weeks).

b) Change in cellular size

The mean size of epithelial cells was $15,124 \pm 4,984$ pixels before treatment and was greatest after 4 weeks of treatment ($5,6167 \pm 13,702$ pixels; **Table 3b**). The mean sizes after 8 and 12 weeks of treatment were $20,631 \pm 8,403$ pixels and $14,247 \pm 8,296$ pixels, respectively, and the size at 16 weeks was less than that at diagnosis in any good responder. In 1 poor responder (patient 7), cell size after 16 weeks of treatment was greater than that before treatment and, in the other poor responder (case 6), cell size after 16 weeks of treatment was much greater than the mean cell size in good responders. The differences in cell size for each treatment period between good responders and poor responders became significant only after 4 weeks of treatment ($p=0.048$). Considering these histological findings, observation of cell size after 4 weeks of treatment is important for predicting the hormonal response to further treatment. After calculating the swelling ratio for each case (**Table 3b**), the average swelling ratio in the 5 good responders was 3.83 but

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Table 2 Physical profiles and serum CA 125 values of the 7 Cases

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7
Age (years)	20	24	27	31	34	36	35
Body height (cm)	169	166	156	153	157	161	163
Body weight (kg)	65	72	118	87	72	90	102
Body mass index (kg/m ²)	22.9	26.1	48.5	37.2	29.1	34.7	38.4
Serum CA125 (U/mL)	6	13	8.7	25.7	22	15.2	13

Table 3 Possible predictors for hormonal response in 7 cases of endometrial adenocarcinoma treated with a high-dose progesterin

Factor (a.-f.)/ Observation period	Patient number						
	1	2	3	4	5	6	7
<i>a. Stroma/Tumor Area (%: mean \pm SD, N=3)</i>							
Before treatment	5 \pm 4	4 \pm 1	5 \pm 1	5 \pm 2	5 \pm 2	6 \pm 3	5 \pm 2
After 4 weeks of treatment	10 \pm 8	11 \pm 3	52 \pm 9	21 \pm 3	29 \pm 7	38 \pm 19	11 \pm 4
After 8 weeks of treatment	42 \pm 10	53 \pm 10	91 \pm 2	62 \pm 20	60 \pm 7	49 \pm 19	22 \pm 10
After 12 weeks of treatment	91 \pm 6	92 \pm 2	95 \pm 1	90 \pm 3	92 \pm 3	51 \pm 24	31 \pm 10
After 16 weeks of treatment	95 \pm 2	98 \pm 2		95 \pm 3	95 \pm 3	62 \pm 20	30 \pm 9
<i>b. Epithelial cell size (pixels: mean \pm SD, N=3-5)</i>							
Before treatment	13,087 \pm 4,077	14,913 \pm 4,044	9,862 \pm 2,239	14,448 \pm 7,615	23,310 \pm 5,109	31,969 \pm 4,750	7,950 \pm 1,157
After 4 weeks of treatment	36,146 \pm 10,409	60,714 \pm 9,592	48,798 \pm 15,080	64,918 \pm 28,135	70,259 \pm 22,700	34,412 \pm 10,987	7,830 \pm 1,707
After 8 weeks of treatment	13,166 \pm 1,845	15,224 \pm 2,878	33,961 \pm 7,717	23,540 \pm 4,138	17,265 \pm 2,395	20,121 \pm 5,128	4,446 \pm 424
After 12 weeks of treatment	8,892 \pm 1,689	8,995 \pm 2,721	8,938 \pm 1,041	27,869 \pm 7,316	16,543 \pm 8,267	25,893 \pm 8,942	18,530 \pm 3,695
After 16 weeks of treatment	8,965 \pm 1,341	11,023 \pm 1,734		8,593 \pm 2,435	9,504 \pm 846	17,588 \pm 4,506	9,202 \pm 1,272
<i>c. Ki-67 nuclear antigen labeling index (%: mean \pm SD, N=3-5)</i>							
Before treatment	42 \pm 9.8	36 \pm 6.8	37 \pm 4.3	33 \pm 8.3	38 \pm 5.7	56 \pm 9	46 \pm 12
After 4 weeks of treatment	4.1 \pm 2.2	7.3 \pm 1.8	1.8 \pm 0.6	1.5 \pm 0.9	1.3 \pm 0.3	61 \pm 18	20 \pm 5.5
After 8 weeks of treatment	5.9 \pm 2.9	6.6 \pm 4.2	0.6 \pm 0.3	3.7 \pm 0.4	1.8 \pm 0.3	77 \pm 25	21 \pm 5.3
After 12 weeks of treatment	4.4 \pm 3.4	2.2 \pm 1.4	0.5 \pm 0.3	6.4 \pm 3.7	5 \pm 2.3	61 \pm 17	31 \pm 8.8
After 16 weeks of treatment	0.5 \pm 0.7	1.1 \pm 0.3		0.4 \pm 0.3	3.5 \pm 2.2	34 \pm 16	17 \pm 6.6
<i>d. Single-stranded DNA labeling index (%: mean \pm SD, N=3-5)</i>							
Before treatment	12 \pm 6	5 \pm 3	5 \pm 2	9 \pm 1	11 \pm 6	15 \pm 2	6 \pm 2
After 4 weeks of treatment	9 \pm 4	1 \pm 1	12 \pm 4	17 \pm 7	4 \pm 1	12 \pm 3	3 \pm 2
After 8 weeks of treatment	8 \pm 4	7 \pm 3	8 \pm 5	10 \pm 2	6 \pm 3	10 \pm 3	6 \pm 2
After 12 weeks of treatment	3 \pm 1	2 \pm 1	10 \pm 3	17 \pm 3	4 \pm 1	4 \pm 1	3 \pm 1
After 16 weeks of treatment	0 \pm 0	1 \pm 1		1 \pm 0	8 \pm 4	5 \pm 2	4 \pm 3
<i>e. ER labeling index (%: mean \pm SD, N=3-5)</i>							
Before treatment	98 \pm 1	79 \pm 9	86 \pm 6	71 \pm 12	95 \pm 3	90 \pm 2	67 \pm 19
After 4 weeks of treatment	24 \pm 6	61 \pm 22	16 \pm 6	89 \pm 10	75 \pm 10	49 \pm 11	81 \pm 8
After 8 weeks of treatment	27 \pm 18	76 \pm 19	25 \pm 12	39 \pm 21	39 \pm 16	92 \pm 3	58 \pm 8
After 12 weeks of treatment	64 \pm 19	71 \pm 10	74 \pm 15	34 \pm 16	6 \pm 2	87 \pm 13	64 \pm 18
After 16 weeks of treatment	15 \pm 8	42 \pm 9		36 \pm 5	20 \pm 10	45 \pm 10	29 \pm 9
<i>f. PR labeling index (%: mean \pm SD, N=3-5)</i>							
Before treatment	87 \pm 8	69 \pm 15	82 \pm 6	79 \pm 14	58 \pm 14	87 \pm 7	80 \pm 10
After 4 weeks of treatment	88 \pm 7	49 \pm 14	51 \pm 15	90 \pm 2	63 \pm 14	98 \pm 4	57 \pm 20
After 8 weeks of treatment	42 \pm 6	50 \pm 16	1 \pm 1	4 \pm 4	10 \pm 5	72 \pm 15	80 \pm 9
After 12 weeks of treatment	35 \pm 15	17 \pm 8	4 \pm 3	47 \pm 31	1 \pm 1	84 \pm 4	34 \pm 18
After 16 weeks of treatment	1 \pm 1	3 \pm 2		28 \pm 21	12 \pm 10	50 \pm 11	35 \pm 13

was 1.08 and 0.98 in the 2 poor responders. Therefore, cellular swelling could be considered a characteristic indicating the hormonal response to treatment.

c) Labeling indices of Ki-67 nuclear antigen, ssDNA, ER, and PR

The Ki-67 nuclear antigen labeling indices of the 5 good responders before treatment were 33% to 42% (mean \pm standard deviation [SD], $37.2\% \pm 2.2\%$), whereas those in the 2 poor responders were 46% and 56% (**Table 3c**). Although there were no statistically significant differences between good responders and poor responders at each time point, the indices after treatment were much higher (20%–77%) for the poor responders than for the good responders (0.4%–7.3%). Therefore, the sharp reduction in this labeling index from diagnosis to after 4 weeks of treatment could indicate a good hormonal response. Although these results show that the Ki-67 nuclear antigen labeling index in poor responders might become high, the tendency could not be more precisely evaluated because of the small number of subjects.

The ssDNA labeling indices were 1% to 17% at either the time of diagnosis or after 4 to 16 weeks of treatment. No clear differences could be recognized either between good responders and poor responders or between treatment times ($p > 0.05$); therefore, this factor is not a good candidate for predicting hormonal response.

The ER labeling indices for both good responders and poor responders were moderately high and ranged from 67% to 98% (mean \pm SD, $84 \pm 12\%$) at the time of diagnosis and decreased in 4 of the 5 good responder and in 1 of the 2 poor responders after 4 weeks after treatment. The labeling indices thereafter increased in some patients and decreased in other patients among both the good responders and poor responders (**Table 3e**). Similarly, because there was no significant difference between the good responders and poor responders at any time point (i. e., all p -values > 0.05), the ER labeling index is not suitable as a predictor of hormonal response.

Before treatment, the PR labeling indices of both good responders and poor responders were moderately high and ranged from 58% to 87% (mean

\pm SD, $77\% \pm 10\%$). After 4 weeks of treatment, the indices were still high and ranged from 49% to 98% (mean \pm SD, $70\% \pm 18\%$) in both good responders and poor responders. Thereafter, the indices of poor responders remained high, whereas those of good responders were more frequently low (**Table 3e**). However, there were no statistically significant differences between the good responders and poor responders at each time point. Therefore, these changes were insufficient for predicting hormonal responses.

Discussion

Conservative therapy with a high-dose progestin for early uterine corpus carcinoma of well-differentiated endometrioid histologic type has technical disadvantages. The endometrial lesion state must be evaluated with such blind methods as curettage from diagnosis to assumed cure. For this reason, good predictors of hormonal response are needed to guarantee that cure is complete and to exclude masked cases of high-risk malignancy that were initially presumed to be amenable to treatment. It does not seem reasonable to include adenocarcinomas of other histologic types, such as the serous type, in a study of hormonal response for endometrioid morphology. However, adenocarcinomas having small foci of other histologic type but otherwise being of endometrioid morphology might be misdiagnosed as pure endometrioid adenocarcinomas, these tumors might then be inappropriately treated with high-dose progestin therapy. Our present study was planned to exclude such cases early during hormonal therapy on the basis of the results of potential predictors of good hormonal response.

Our previous study found that the first change observed during high-dose progestin therapy was the swelling of neoplastic epithelial cells with an associated decrease in mitotic index, increased predecidualized stroma, and little change in the apoptotic index⁴. However, these changes were based on detailed microscopic observation and were extremely qualitative in character. Therefore, to confirm our previous result we attempted to analyze

these changes more quantitatively in the present study by means of immunohistochemistry and computer software analysis.

First, we attempted to measure the relative area occupied by stroma in each tumor. When we examine a tumor microscopically, we usually make qualitative evaluations of the proportions of the epithelial and stromal components on the basis of what can be seen but do not quantitatively verify how much stroma is present. Therefore, we started measuring the stromal areas to see how they changed after treatment. The stroma at diagnosis occupied less than 6% of the whole tumor area in either good responders or poor responders and increased from 10% to 52% after 4 weeks of treatment. In good responders, the stroma came to occupy more than 95% of the whole tumor area after 12 to 16 weeks of treatment, whereas in poor responders the stroma occupied only 30% and 62% of the tumor even after 16 weeks of treatment. Although this ratio was first thought to be a potential predictor of hormonal response, consideration of the histopathological meaning of epithelial and stromal components has suggested that this ratio indicates only the final outcome of the hormonal response at a given time and cannot predict the subsequent response. Therefore, this factor appears to be an inappropriate candidate for a predictor of hormonal response.

We next measured the average cell size of the glandular epithelium for each biopsy specimen. Because cellular swelling probably reflects the degenerative process leading to necrosis through secretory change, the change in cell size must be a marker of cell damage⁴⁶. Accurately measuring cell size was difficult, but evaluating relative size was simple with computer software programs. Adobe Photoshop can be used to color areas of interest, and I2A is a free software program that allows areas of a given color to be measured in pixels.

In this study, we colored stromal cells blue and measured blue areas using the I2A program. Although stromal areas were measured in pixels, we could still evaluate relative size for comparison.

Two methods of autocalculation for immunostaining labeling indices have been

reported^{7,8}. Amin et al counted MIB-1-stained glioma cells automatically by appropriately adjusting the threshold level and minimal particle size in the NIH imaging program⁷. Although this method does not require manual procedures and takes little time, a problem is that the above adjustments affect the overall results. Furthermore, endothelial cells or inflammatory cells were counted, and neighboring overlapping cells were not counted⁷.

In contrast, the method proposed by Ide et al. does not differ fundamentally from cell counting with microscopy but has the distinct advantage of avoiding counting errors, such as overlapping or missing data⁸. Because inflammatory cells were found in various areas of the specimens in the present study, we decided to use the method of Ide et al.

Ki-67 is a nuclear antigen recognized by the anti-MIB-1 antibody. Ki-67 is related to cell proliferation, and its labeling index indicates proliferative ability. In the present study, Ki-67 labeling index markedly decreased in good responders but showed little change in poor responders. This tendency was the same as our previous result for the mitotic index and supports the idea that mitotic arrest might occur owing to the effects of a progestin⁴.

Apoptosis can be demonstrated with DNA fragmentation and the expression of apoptosis regulatory proteins (e.g. caspase 3, Bcl-2, and Bax) and can generally be detected with the terminal deoxyribonucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick-end labeling method (TUNEL assay) and immunochemistry for ssDNA or caspase 3⁹. Because Groos et al. have reported that the antibody detecting formamide-denatured ssDNA in apoptotic cells was both more appropriate and more reliable than the TUNEL assay¹⁰, we detected apoptosis with immunochemical methods and mouse polyclonal antibody for ssDNA¹⁰. As in our previous report, we did not find any definite tendency for change in the apoptotic index from direct observation of H&E-stained slides or in the ssDNA apoptotic activity⁴.

Ehrlich et al has studied the use of ER or PR or both as predictors of progestin response and has reported that decreased PR activity is associated

with increased anaplasia in irradiated tumors, whereas Yamazawa et al and Yang et al have reported the usefulness of PR as a predictor of the response of endometrial adenocarcinomas to high-dose progestin therapy¹¹⁻¹³.

Although our finding that ER decreased in 5 of 7 cases during progestin treatment might be due to the effect of the progestin, there was no relationship between this effect and the PR labeling index at the time of diagnosis. Although the PR labeling index might decrease over the long term in good responders, this result has an opposite meaning, according to Erlich et al¹¹. The indices of poor responders should have decreased owing to anaplasia if the reasoning of Erlich et al. is applied. However, the contradictory results of our study might be explained by the heterogeneity of PR distribution in human endometrial adenocarcinomas, as previously reported by Zaino et al¹⁴.

Finally, the histological changes we observed might not follow the linear change of the malignant epithelium, and the disappearance of invasive carcinoma at subsequent curettage could not be purely due to the effects of hormonal treatment but also due to the removal of the tumor by means of curettage. However, glandular changes observed after 4 weeks of treatment should definitely have occurred in the malignant epithelium, because we did not remove the tumor completely at the first curettage. In other words, we cannot describe the mechanism of disappearance, but we would like to clarify the phenomenon of disappearance until cure during this kind of high-dose progestin therapy. It is because we could not evaluate histological change only through specimens obtained through measures other than curettage. Therefore, the variables observed at an early stage of treatment might be especially important in clinical practice.

In conclusion, epithelial cell swelling, which was identified in our previous study as a good predictor of hormonal response on the basis of subjective examination, was, in the present study, semiquantitatively and more objectively confirmed to be such a predictor. Among the candidates semiquantitatively analyzed as predictors associated with cell proliferation, apoptosis, or hormone

receptors, the marked decrease in the Ki-67 nuclear antigen (MIB-1) labeling index after 4 weeks of treatment proved to be the best predictor of the hormonal response to high-dose progestin therapy. These findings, therefore, support the results of our previous study, which were also obtained with detailed microscopic observation of H&E-stained specimens. While the number of cases studied was limited, this study suggests a more reliable way to predict hormonal response and should be followed up with additional case studies.

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