

Acta Medica Okayama

Volume 46, Issue 2

1992

Article 8

APRIL 1992

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Proliferative activity in normal endometrium and endometrial carcinoma measured by immunohistochemistry using Ki-67 and anti-DNA polymerase alpha antibody, and by flow cytometry.*

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Abstract

The proliferative activity of various parts of normal and malignant endometrium was evaluated using an immunohistochemical approach and flow cytometry (FCM). The two monoclonal antibodies, Ki-67 and anti-DNA polymerase alpha antibody (anti-poly alpha antibody) were used to detect the proliferative activity of cells, and the percentage of the Ki-67 and anti-poly alpha positive cells were measured. Proliferative indices (PI; percentage of S and G2M phase) and DNA ploidy were measured by FCM. Normal endometrial specimens from 29 patients with benign diseases were used and three different parts (fundus, middle, and low part of the uterus) were examined. In the proliferative phase of normal endometrium, there was no significant difference in the proliferative activity in the three parts. In 20 patients with endometrial carcinomas with myometrial invasion, tissues were taken from the myometrial invasive site and the central part of the tumor tissue. In the cases of endometrial carcinoma, the myometrial invasive site had a higher proliferative activity than central part of the tissue. The proliferative activity measured by the immunohistochemistry was correlated with the histological grade of malignancy, but it was not consistent with PI by FCM. This suggests that the proliferative activity measured by the immunohistochemistry is independent of flow cytometric PI.

KEYWORDS: endometrial carcinoma, immunohistochemistry, monoclonal antibody Ki-67, monoclonal antibody anti-DNA polymerase ?, flow cytometry(FCM)

*PMID: 1575057 [PubMed - indexed for MEDLINE]

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Proliferative Activity in Normal Endometrium and Endometrial Carcinoma Measured by Immunohistochemistry Using Ki-67 and anti-DNA Polymerase α Antibody, and by Flow Cytometry

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The proliferative activity of various parts of normal and malignant endometrium was evaluated using an immunohistochemical approach and flow cytometry (FCM). The two monoclonal antibodies, Ki-67 and anti-DNA polymerase α antibody (anti-poly α antibody) were used to detect the proliferative activity of cells, and the percentage of the Ki-67 and anti-poly α positive cells were measured. Proliferative indices (PI; percentage of S and G2M phase) and DNA ploidy were measured by FCM. Normal endometrial specimens from 29 patients with benign diseases were used and three different parts (fundus, middle, and low part of the uterus) were examined. In the proliferative phase of normal endometrium, there was no significant difference in the proliferative activity in the three parts. In 20 patients with endometrial carcinomas with myometrial invasion, tissues were taken from the myometrial invasive site and the central part of the tumor tissue. In the cases of endometrial carcinoma, the myometrial invasive site had a higher proliferative activity than central part of the tissue. The proliferative activity measured by the immunohistochemistry was correlated with the histological grade of malignancy, but it was not consistent with PI by FCM. This suggests that the proliferative activity measured by the immunohistochemistry is independent of flow cytometric PI.

Key words : endometrial carcinoma, immunohistochemistry, monoclonal antibody Ki-67, monoclonal antibody anti-DNA polymerase α , flow cytometry (FCM)

Endometrial carcinoma is known to originate mainly in or near the fundus of the uterine body. This suggests that the endometrium of the fundus has some properties that enable carcinoma to develop. It is also known that the proliferative activity of normal endometrium is as high as endometrial carcinoma (1). We attempted to

clarify these special properties of the fundus from the point of view of tissue proliferative activity. We examined the proliferative activity in three different parts (fundus, middle, and lower part) of the normal endometrium and endometrial carcinoma. In endometrial carcinoma, the proliferative activity in the central part of tumor tissue and myometrial invasive sites were investigated.

Proliferative activity was evaluated as growth

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fraction (GF) by immunohistochemistry with monoclonal antibody Ki-67 and anti-DNA polymerase α (anti-poly α) antibody. In endometrial carcinoma, DNA ploidy and proliferative indices (PI; percentage of S and G2M phase) measured by flow cytometry (FCM) were used for DNA content analysis in addition to immunohistochemical approach. The correlations of GF to clinical stage, histological grade, DNA ploidy and PI were also evaluated.

Materials and Methods

Materials. Twenty-nine fresh normal endometrial specimens were surgically removed from the patients with uterine myoma. They had well-established histories of regular menstrual cycles. Twenty-one cases were in the proliferative phase and 8 in the secretory phase. Among the proliferative cases, 3 were in early proliferative, 6 in the middle, and 12 in the late phases. Small specimens (about 5mm³ blocks) were taken from the three parts (fundus, middle and low part) of the uterine body. The fundus was defined as the site of the fundus in the uterine body. The low part was at a site about 5mm above the internal os, and middle part. Endometrial dating of the specimens was diagnosed with hematoxylin-eosin stained preparations before immunostaining.

The specimens of endometrial carcinoma were obtained by surgery from 20 patients. The specimens were taken from the myometrial invasive site and the central part of tumor tissue, and excessive necrotic and hemorrhagic areas were excluded. All specimens were examined within two weeks after removal.

Surgical staging and histological grade classification were performed according to the new FIGO criteria (2).

Ki-67 staining. Specimens were embedded in OCT compound immediately after surgical resection and stored at -80°C . Six micrometers thick cryostat sections were fixed in acetone for 10 min at 4°C and air dried on gelatin-coated slides for 15 min. The sections were exposed to 10 % normal horse serum to prevent non-specific binding of the antibody after blocking endogenous peroxidase by 0.3 % hydrogen peroxide (H_2O_2) in methanol. The avidin-biotin complex (ABC) method was employed for Ki-67 staining using the Vectastain ABC kit (Vector Labs. Burlingame, CA, USA). The sections were first incubated with monoclonal antibody (MoAb)

Ki-67 (25-fold diluted with phosphate-buffered saline (PBS) containing 0.5 % Tween 20) for 1 h at room temperature, followed by incubation with the avidin-biotin-peroxidase complex. Labeling was developed with a diaminobenzidine hydrogen peroxide substrate (DAB, Wako, Osaka, Japan) and sections were lightly counterstained with Mayer's hematoxylin. Ki-67 positivity was defined as the percentage of Ki-67 positive cells in about 1,000 cells.

Anti-DNA polymerase α antibody staining. Six micrometers thick sections were fixed in 4 % paraformaldehyde (PFA) for 30 min at 4°C . After immersion in 0.3 % H_2O_2 in PBS, they were rinsed three times in PBS. Anti-poly α antibody staining was performed using peroxidase anti-peroxidase (PAP) technique with MoAb anti-poly α (CL22-2-42B) purchased from MBL (Medical and Biochemical Lab., Nagoya, Japan). Using the MBL kit, after incubation with the primary antibody, slides were incubated with rabbit anti-mouse IgG (bridging antibody) followed by incubation with mouse PAP complex. The percentage of cells positive for anti-poly α antibody was determined microscopically by counting approximately 1,000 cells.

Flow cytometry (FCM). Adjacent portions of the same specimens used for immunostaining were analyzed by FCM. The small tissue fragments were minced with scissors, and the cells were fixed in 2 % PFA for 20 min at 4°C and washed twice in PBS (–) solution. These cells were then treated with PBS (–) containing 0.1 % Triton X-100 and 0.1 % RNase (Sigma Chemical, St. Louis, USA), and stained with propidium iodide (Calbiochem-Behring, Co.). The final concentration of propidium iodide was adjusted to 50 mg/ml. Samples were analyzed on an EPICS V flowcytometer (Coulter Co., USA) where at least 10,000 nuclei were counted. The flowcytometer was adjusted to the G0G1 peak channel number of human monocytes before measuring DNA contents. Human monocyte suspensions were prepared from peripheral blood using Ficoll-paque density gradient centrifugation. DNA ploidy was classified into diploidy and aneuploidy according to the Committee on Nomenclature of the Society for Analytical Cytology (3). Histogram analysis was performed with a standard program PARA 1 (a Gaussian fractionation procedure). The percentages of cells in S and G2M phases were summed to define the proliferative index (PI).

Statistical analysis. Growth fraction measured with Ki-67 and anti-poly α antibody and flow cytometric PI were not normally distributed. Therefore, correlations between the GF and PI were measured with

Pearson's correlation coefficient. Tests of the significant difference between paired samples were performed using the Student's *t* test.

Results

The results of FCM analysis of twenty-nine benign and twenty malignant specimens are summarized in Table 1. The twenty-nine normal endometrial specimens were all found to be di-

ploid. Six out of 20 endometrial carcinoma samples were shown to be aneuploid. Aneuploidy was found in 1 of 9 well-differentiated groups (Grade 1), and in 3 of 8 moderately differentiated groups (Grade 2) and in 2 of 3 poorly differentiated groups (Grade 3). The frequency of aneuploidy tended to rise according to the elevation of histological grade.

In the normal proliferative endometrium, the percentage of Ki-67 positive cells was 25.9 ± 6.3 (mean $\pm$ SD) % in the fundus, 26.0 ± 3.3 % in

Table 1 Results of flow cytometric analysis of benign and malignant endometrial specimens

Histological features	Number of specimens		
	Diploidy	Aneuploidy	Total
Benign	21	0	21
Proliferative	8	0	8
Secretory	8	0	8
Carcinoma			
Well-differentiated	8	1	9
Moderately differentiated	5	3	8
Poorly differentiated	1	2	3

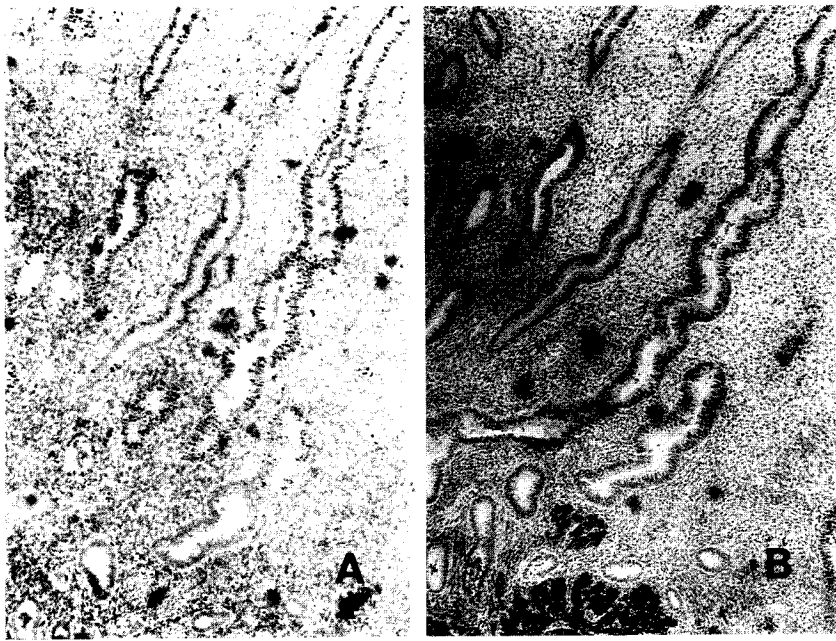


Fig. 1 A: Ki-67 staining in the late proliferative phase. B: anti-poly α antibody staining in the late proliferative phase (adjacent to the lesion of Ki-67 staining). The degree of anti-poly α staining was almost same as that of Ki-67 staining.

the middle and $25.5 \pm 5.8\%$ in the low part of the uterus (Fig 1). The percentage of anti-poly α antibody positive cells was $27.2 \pm 5.8\%$, $24.0 \pm 2.9\%$, and $24.8 \pm 4.0\%$, respectively. Among the three parts of normal endometrium, there were no significant differences in Ki-67 and anti-poly α antibody positive rates.

A Grade 1 endometrial carcinoma and its Ki-67 and anti-poly α antibody immunostains are shown in Fig 2. Multiple stained spots were

visible in the nuclei of cells stained with Ki-67. Nuclei were diffusely stained with anti-poly α antibody.

In endometrial carcinoma, the percentage of Ki-67 positive cells was $36.9 \pm 9.9\%$ in the myometrial invasive site and $19.4 \pm 5.6\%$ in the central tumor site. The range of anti-poly α antibody positive cells was $37.9 \pm 9.4\%$ and $20.8 \pm 5.5\%$ in these two sites, respectively. Both averages of Ki-67 and anti-poly α antibody

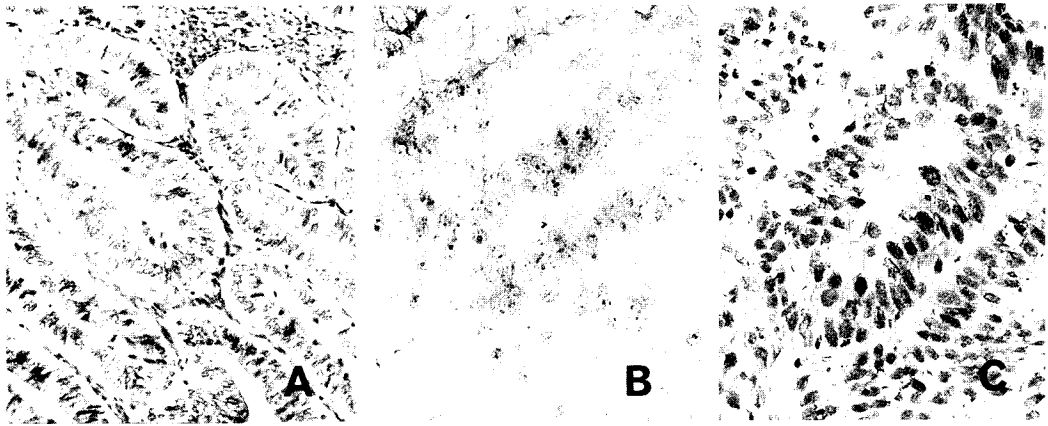


Fig. 2 A: Grade 1 endometrial adenocarcinoma. B: Ki-67 staining. Multiple spotty stainings are visible in the nuclei. C: anti-poly α antibody staining. Nuclei are diffusely stained.

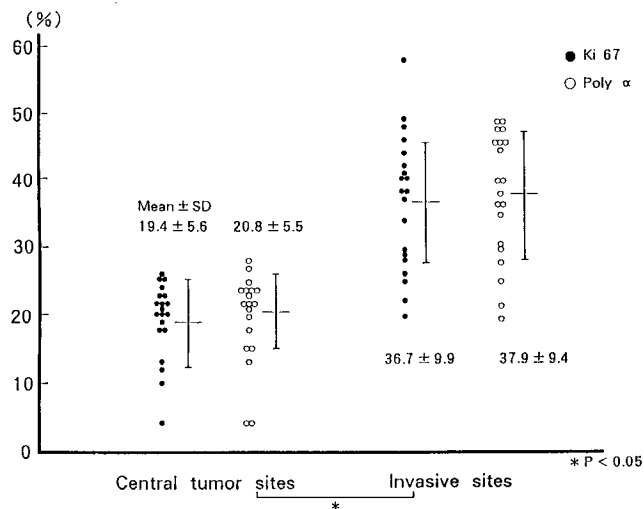


Fig. 3 The percentage of Ki-67 and anti-poly α antibody positive cells for central tumor and invasive sites (horizontal lines represent mean values).

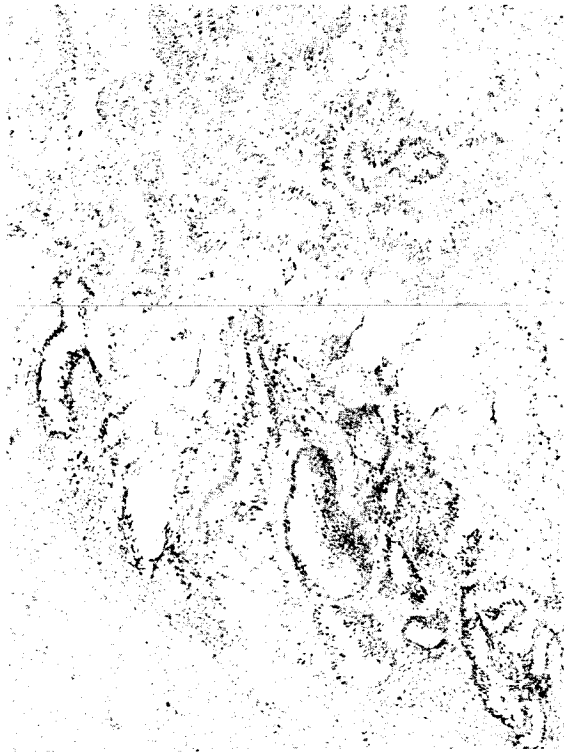


Fig. 4 Typical immunostaining patterns with Ki-67 in endometrial carcinoma. Reactivity with Ki-67 is higher in the myometrial invasive site (bottom) than in the central tumor site (top).

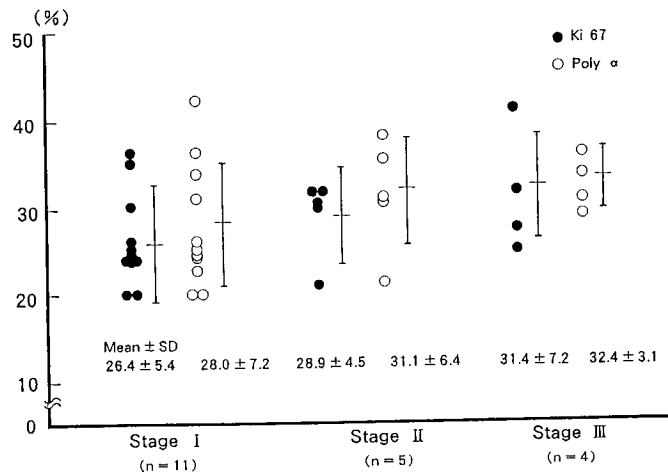


Fig. 5 The percentage of Ki-67 and anti-poly α antibody positive cells in relation to clinical stage.

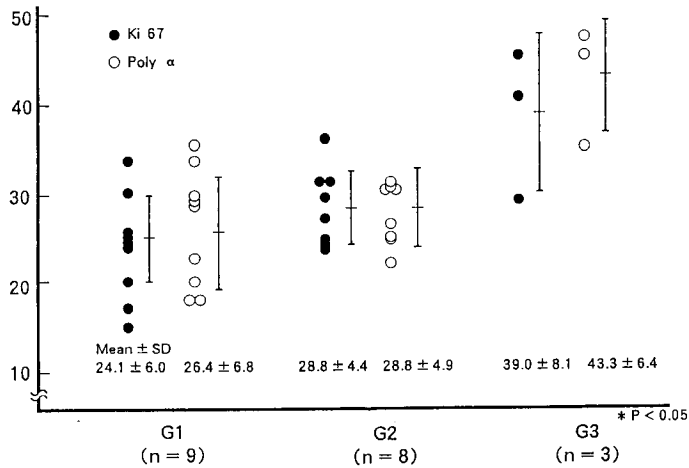


Fig. 6 The percentage of Ki-67 and anti-poly α antibody positive cells in relation to histological grade.

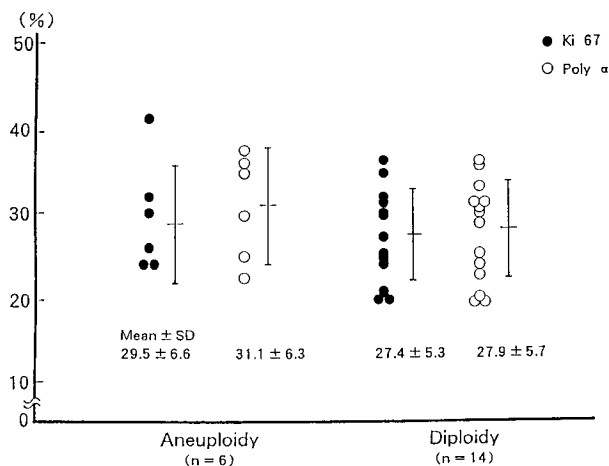


Fig. 7 The percentage of Ki-67 and anti-poly α antibody positive cells in relation to diploidy and aneuploidy.

positive rates showed the higher levels in the myometrial invasive site than in the central part of the tumor tissue (Fig. 3). Fig. 4 shows that, according to Ki-67 staining, the myometrial invasive site had a higher proliferative activity than the central part of the tumor site.

The average of Ki-67 positivity was 26.4 % in clinical stage I, 28.9 % in stage II and 31.4 % in stage III (Fig. 5). The average of anti-poly α antibody positive rates was 28.0 %, 31.1 %, and

32.4 %, respectively. There was no significant correlation between the GF with two MoAb and clinical stage.

Fig. 6 shows the mapping of the GF values in different histological grades. The means of Ki-67 positive cells were 24.1 % in Grade 1, 28.8 % in Grade 2 and 39.0 % in Grade 3. The mean of anti-poly α antibody positive cells was 26.4 %, 28.8 % or 43.3 % for the respective grades. Grade 3 indicated a higher level of the GF than

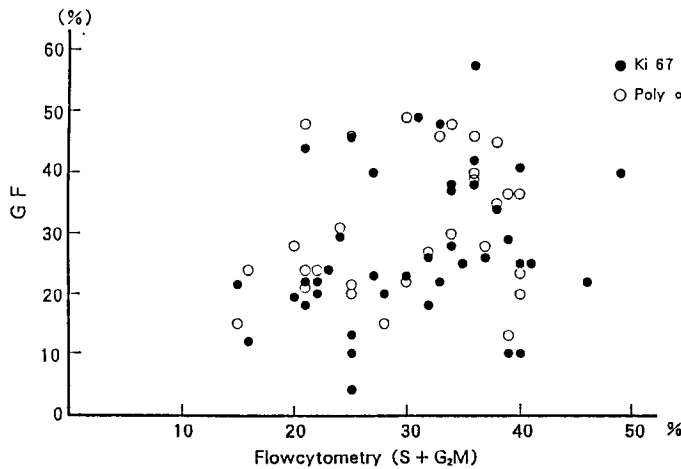


Fig. 8 Plot of percentage of Ki-67 and anti-poly α antibody positive cells against flow cytometric proliferative indices.

Grades 1 and 2. We could not detect any difference between Grades 1 and 2 with regard to their immunostaining properties.

Ki-67 positivity ranged from 24.0 % to 41.3 % with a mean of 29.5 % in aneuploid tissue and from 20.1 % to 36.5 % with a mean of 27.4 % in diploid tissue (Fig. 7). The range of anti-poly α positive cells was 22.5 % to 38.0 % with a mean of 31.1 % in aneuploid tissue and 20.0 % to 36.5 % with a mean of 27.9 % in diploid tissue. The highest Ki-67 positivity was observed in aneuploid tissue. However, the values of the two immunostainings were not significantly different.

Distributions between GF and flow cytometric PI are shown in Fig. 8. There was a poor correlation between the GF and flow cytometric PI ($r = 0.28$).

Discussion

It is widely recognized that the S phase fraction of cell cycle reflects well the proliferative activity of tumors. Recently, S-phase cell-labeling with bromodeoxyuridine (BrdU) has been used to investigate the proliferative activity in many malignant tumors by immunostaining

methods using an anti-BrdU antibody (4-7). The monoclonal antibodies to a proliferative cell nuclear antigen and to DNA polymerase α have been produced recently and used to measure GF. Ki-67 (8) and anti-poly α antibody (9), which are commercially available, have been used for cell cycle analysis by immunohistochemistry. The percentages of Ki-67 positive cells and the anti-poly α antibody positive cells in total cells were reported to be parallel to the BrdU labeling index (10,11). Compared with the BrdU labeling method, Ki-67 and anti-poly α antibody staining methods do not require the injection of antigens and DNA denaturation process. Thus, the Ki-67 and anti-poly α antibody stainings are preferable to BrdU labeling to assess the cellular proliferative activity. Almost the same percentage of Ki-67 and anti-poly α antibody positive cells was observed in the same material in our study.

The GF of the gland cells in the three different parts of the uterus was almost the same. But in fact, the fundus of normal endometrium is thicker than the other parts and grows about 7 to 10 mm thick in the late proliferative phase. This difference in the thickness of endometrial glands has generally been recognized to depend on a higher hormonal reaction in the fundus of

endometrium (13). As glands of various parts have the similar proliferative activity, this difference in the thickness may be related to other factors, such as the difference in hormone receptor concentrations and the proportion between glands and stroma. The area where carcinoma originates is thought not to be associated with the proliferative potential of endometrium.

There have been few reports on the proliferative activity of different sites of endometrial carcinoma, *e.g.*, the central tumor site and the myometrial invasive site. The myometrial invasive site showed higher GF than central part of the tumor tissue ($p < 0.05$).

With regard to histological grade, Mushika *et al.* (14) reported that Grade 2 endometrial carcinoma had a higher average of anti-poly α antibody positive cells than Grade 1 in 19 cases. The percentage of Ki-67 and anti-poly α antibody positive cells in Grade 1 was not significantly different from Grade 2, but was higher in Grade 3 than Grades 1 and 2 in this study. We took the tissues with myometrial invasion, but Mushika *et al.* (14) used the tissues with or without myometrial invasion. This difference in the materials studied might cause the difference in their and our results.

Some investigators indicate that DNA ploidy is a better prognostic indicator than the histological grade of tumors, the depth of invasion, and the concentration of estrogen receptor in endometrial carcinoma (15,16). Sasaki *et al.* (17) reported that DNA ploidy was not closely related to Ki-67 positivity in human brain tumors. Our results were in agreement with their results.

There are few reports that the Ki-67 and anti-poly α antibody positive rates are compared with PI measured by FCM. James *et al.* (18) reported that there was a poor correlation between PI and S-phase labeling in endometrial carcinoma. In other carcinomas, such as breast carcinoma, non-Hodgkin's lymphoma and malignant soft tissue tumors, no significant correlation was observed between the percentage of Ki-67 positive cells and the higher PI (19-21).

PI measured by FCM shows the fraction of cells from S to G2M phases. On the other hand, the GF measured with Ki-67 and anti-poly α antibody demonstrates the fraction of the cells from G1 to G2M phase in the total cell population. Theoretically, the fraction of G1 phase plus PI equal GF. Schwartz *et al.* (19) suggested that G1 phase content may influence the correlation between Ki-67 positivity and PI, because the length of this phase is known to be the most variable part of each cell cycle.

In our study, the GF showed no specific correlation with the clinical stage, histological grade, DNA ploidy and PI. The GF with Ki-67 and anti-poly α antibody positive cells and PI measured by FCM should be considered to play independent roles as indicators for proliferative activity and prognostic values in endometrial carcinoma. Sufficient clinical follow-up will provide the data necessary to assess the significance of those indicators for endometrial carcinoma.

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Received November 26, 1991; accepted January 28, 1992.