Hirschsprung's disease, and papillary thyroid carcinoma[2-4]. In our previous study RET was expressed in all neuroblastomas developed from 11 tumor cell lines and 29 fresh tumor cells[5]. Glial cell line-derived neurotrophic factor (GDNF) knock-out mice show phenotypes similar to those of RET knock-out mice[6-8], implying that RET is a functional receptor for GDNF. Because the GDNF receptor itself has no cytoplasmic domain serving as a signal transducer, the tyrosine kinase domain of RET has been proposed to function as a transmitter of the biological signals triggered by GDNF[9].

Moreover, we already obtained a monoclonal antibody to the RET extracellular domain (NBL-1)[10]. For tumor-cell targeting, monoclonal antibodies are frequently used. In addition, several novel peptides that can bind specific molecules and cells have been identified[11-13].

Park et al. have reported that a rationally designed anti-HER2/neu peptide can inhibit the growth of breast cancer cells *in vitro* and *in vivo*[13], suggesting that targeting peptides can mimic the activities of monoclonal antibodies. In the treatment of breast cancer trastuzumab (trade name : Herceptin) is a humanized recombinant monoclonal antibody that recognizes the extracellular domain of the HER2 transmembrane protein. Trastuzumab was the first clinically applied immunologic target specific drug. Its development represents a model for integrating new agents[14,15]. Therefore, the identification of tumor-associated cell surface antigens is important for the development of tumor-targeted antibody therapy[15].

The aim of this study was to confirm the expression of the RET protooncogene in patients with ovarian cancer and to establish the effective ovarian tumor targeting diagnosis and therapy using the anti-RET monoclonal antibody.

MATERIALS AND METHODS

Cell lines

Two cell lines derived from human ovarian cancer were used. The 2008 cell line[16] was kindly provided by Dr. Howell, and the A2780 cell line[17] was provided by Dr. Ozds. Both NB-39-nu cells (human neuroblastoma cells)[18] and HL60 cells (human promyelocytic leukemia cells) were used as RET positive controls.

Tissue samples

Ovarian cancer tissue samples were resected from 82 patients (mean age, 48±11 years) who had been admitted to The Jikei University School of Medicine from 1989 through 1999.

The clinicopathologic diagnoses of ovarian tumors were ovarian cystadenoma (87 cases ; mean age, 38±13 years) and ovarian cystadenomas with borderline malignancy (15 cases ; mean age, 40±15 years). All histological diagnoses were reviewed according to established morphological classification criteria of the World Health Organization. Overall survival was defined by the interval from the first surgery to death.

Reverse transcriptase-polymerase chain reaction

Total tissue RNA was isolated from 2 ovarian cancer cell lines by using the RNeasy Mini kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. Two micrograms of each RNA sample was subjected to complementary DNA synthesis with an Omniscript RT kit (Qiagen) according to the manufacturer's protocol. The polymerase chain reaction (PCR) was subsequently performed to analyze the expression of RET by using 5′<AGATCCTGGAGGATCCAAAG<3′ and 5′<GTATTTGGCGTACTCCACGA<3′ as forward and reverse primers, respectively. The PCR was performed using 1 μl of template, 0.5 μl of each 10 μM primer, 0.1 μl of Taq DNA polymerase (TaKaRa, Tokyo, Japan), 0.5 μl of 10 mM deoxyribonucleoside triphosphate, 2.5 μl of 10×buffer, and 19.9 μl of purified and ionized water (MilliQ, Millipore Corp., Billerica, MA, USA), in a total volume of 25 μl. The thermal cycler conditions used were 94°C for 5 minutes followed by 40 cycles at 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, and a final extension step at 72°C for 5 minutes. The PCR products were visualized with ethidium bromide staining after separation over a 1% agarose gel.

Characterization of the RET protooncogene in ovarian cancer cell lines

Exons 10, 11, and 13 to 16 of the RET protooncogene, which are the most common mutation sites of the RET protooncogene in other diseases, were analyzed in cells of the 2008 cell line[19]. Briefly, each exon was amplified with PCR and reported primers[20] and then cloned into the pTOPO

vector (Invitrogen, Tokyo, Japan). Several independent clones were subjected to sequence analysis with an automated sequencer (Prism 370, Applied Biosystems, Foster City, CA, USA).

Immunohistochemistry

Immunohistochemical staining was performed as follows. The tissues were fixed immediately with buffered 10% formalin for 24 to 72 hours, dehydrated, and embedded in paraffin. Four-micrometer-thick sections were cut and deparaffinized in ethanol and xylene. Immunohistochemical staining was performed with an immunoperoxidase avidin-biotin conjugate system, with diaminobenzidine and hydrogen peroxide as the substrate and with hematoxylin as the counterstain. Slides were rinsed in a phosphate buffer (pH 7.4) for examination. The sections were incubated with a primary polyclonal anti-RET antibody[21]. All primary antibodies were titrated by dilution (1 : 500 with anti-RET polyclonal antibodies) to obtain optimal intensity of specific staining with minimal nonspecific background reactivity. The secondary antibody was a biotinylated horse anti-mouse immunoglobulin (Zymed, South San Francisco, CA, USA) for use with primary polyclonal antibodies. Negative controls initially consisted of tissue processed without inclusion of the primary antibody. The slides were examined with light microscopy and the intensity of immunostaining was evaluated. The intensity of immunostaining in all slides was classified into 4 levels : level 0 for negative staining, level 1 for lower intensity, level 2 for moderate intensity, and level 3 for the highest intensity. To simplify the results, intensity levels 0 and 1 were defined as negative staining, and levels 2 and 3 were defined as positive staining, except for cases in which the number of stained cells in levels 2 and 3 were less than 50% of all cells in a slide.

Statistical analysis

Fisher's exact test were used to compare the RET-positivity rate among patients with different histologic diagnoses (cystadenoma, cystadenoma with borderline malignancy, and cancer). All statistical analyses were performed with a statistical software program (SAS version 9.1, SAS Institute, Cary, NC, USA). A p value of <0.05 was considered to indicate significance.

Results

Expression and characterization of the RET protooncogene in ovarian cancer cell lines

Synthesized complementary DNAs from 2 cell lines

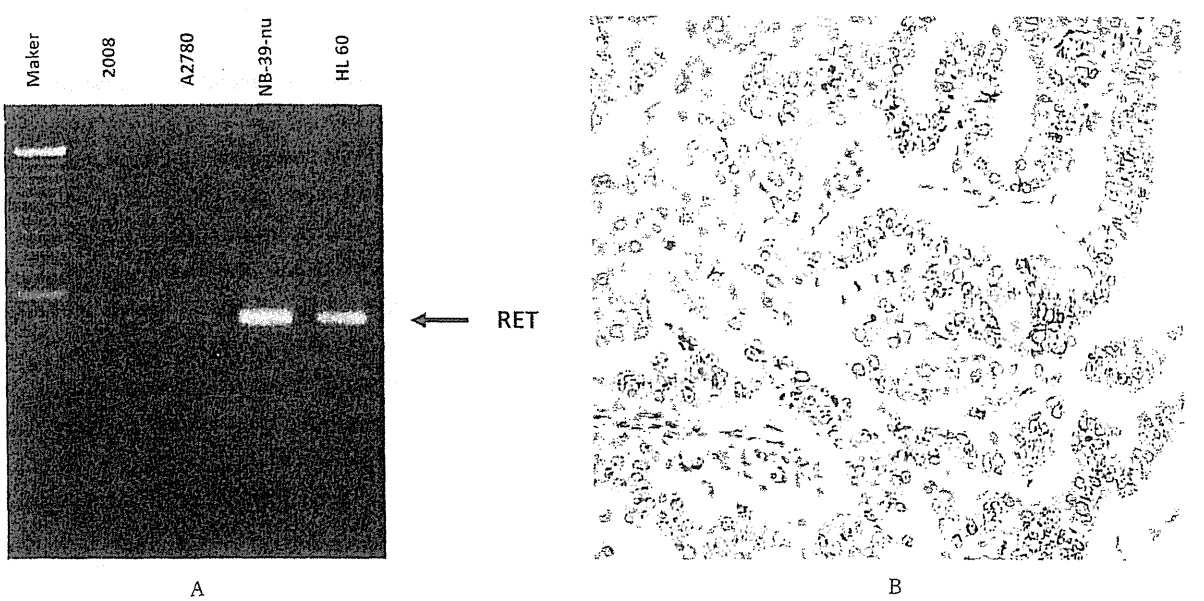

Fig. 1. RET protooncogene expression in ovarian cancer. A, Reverse trascriptase-PCR with primers RET yielded an expected band of 300 bp in 2008 cells and A2780 cells. Both NB-39-nu cells and HL 60 cells served as positive controls. B, Immunohistochemical analysis of ovarian cancer showing cytoplasmic staining for RET.

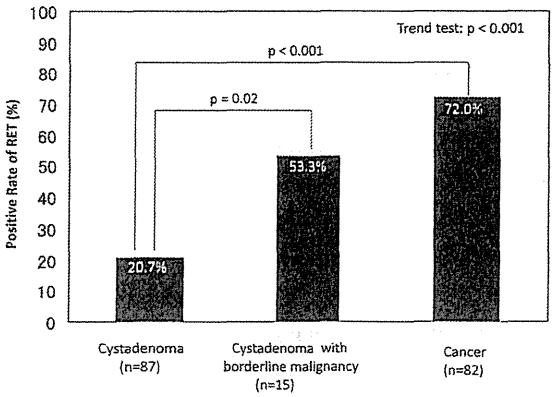

Fig. 2. RET expression in relation to histological type. The rates of RET staining in 3 histologic types : ovarian cystadenoma, cystadenoma with borderline malignancy, and ovarian cancer.

Table 1. Correlation between RET expression and clinicopathologic features in Ovarian Cancer ($n=82$)

Clinicopathologic Parameters	No. of Patients	RET positive rate
FIGO stage		
I	45 (54.9%)	84.4%
II	7 (8.5%)	71.4%
III	26 (31.7%)	53.9%
IV	4 (4.9%)	50.0%
Histologic type		
Serous	25 (30.5%)	48.0%
Endometrioid	10 (12.2%)	70.0%
Mucinous	18 (21.9%)	100%
Clear cell	29 (35.4%)	75.9%
Grade		
1	24 (29.3%)	79.2%
2	46 (56.1%)	71.7%
3	12 (14.6%)	58.3%
Age		
50 >	45 (54.9%)	77.8%
50 ≦	37 (45.1%)	64.9%

(2008 cells, A2780 cells) were successfully amplified with RET primers. The size of PCR products was 300 bp (Fig. 1A). To characterize the RET protooncogene in 2008 cells, PCR amplification followed by nucleotide sequencing was peformed. Analysis of exons 10, 11, and 13 to 16 revealed no mutations (data not shown).

Immunohistochemical staining of ovarian tumors for RET

Immunohistochemical staining for RET proteins was found predominantly in the cytoplasm, but also in the nucleus (Fig. 1B). Among the 82 cases of ovarian cancer (Table 1), the frequency of RET expression was high in mucinous cystadenocarcinoma (18 of 18 cases ; 100%), clear cell adenocarcinoma (22 of 29 cases ; 75.9%), and endometrioid adenocarcinoma (7 of 10 cases ; 70.0%). According to disease stage, the frequency of RET-positive cases was 84.4% (38 of 45 cases) in stage I, 71.4% (5 of 7 cases) in stage II, 53.9% (14 of 26 cases) in stage III, and 50% (2 of 4 cases) in stage IV. According to histological grade, the rate of RET-positive staining was 79.2% in grade 1, 71.7% in grade 2, and 58.3% in grade 3. According to histological type, the rate of RET expression was 72.0% (59 of 82 patients) in ovarian cancer, 53.3% (8 of 15 patients) in ovarian cystadenoma with borderline malignancy, and 20.7% (18 of 87 patients) in ovarian cystadenoma (Fig. 2).

Correlation of RET expression with clinicopathologic characteristics

The rate of RET expression (Fig. 2) was significantly higher in ovarian cancer ($p<0.001$) or ovarian cystadenoma with borderline malignancy ($p=0.02$) than in ovarian cystadenoma.

DISCUSSION

The prognosis of advanced ovarian cancer is poor, with a 5-year survival rate of 30% to 35%, because most cases are not diagnosed until the advanced stage. Furthermore, if the first-line chemotherapy is not effective against ovarian cancer, cancer will continue to grow and spread. Mucinous cystadenocarcinoma and clear cell adenocarcinoma have been reported to show chemoresistance. Our present study has found high expression of the RET protooncogene in mucinous cystadenocarcinoma and clear cell adenocarcinoma. However, the relation between chemoresistance and expression of the RET protooncogene is unclear.

Several research efforts have focused on the identification of new biological markers of prognosis in ovarian cancer. Activation of oncogenes, such as HER-2, and inactivation of onco-suppressor genes, such as p53, have been used in an attempt to assess the prognosis of ovarian cancers[22]. Moreover, in vitro and in vivo studies have ob-

tained some evidence concerning genes related to cellular apoptosis in ovarian carcinogenesis, such as Bcl-2 and p53, and the resistance to chemotherapy in ovarian cancer[23,24]. Aberrations of the proteins produced by these genes are frequently observed in ovarian cancer. However, the degree of protein aberration does not correlate with prognosis.

Ovarian cancer, especially endometrioid adenocarcinoma and clear cell adenocarcinoma, often co-exist with endometriosis. The potential for carcinogenesis, such the change to endometrioid adenocarcinoma or clear cell adenocarcinoma, cannot be ruled out in any case of endometriosis, because no marker is available for identifying this change. The present study is, to our knowledge, the first to examine the expression of RET in a wide spectrum of ovarian tumors. In this study we have found that RET is frequently expressed in endometrioid adenocarcinoma (70%) and clear cell adenocarcinoma (75.9%). Thus, RET expression may be useful for assessing the potential for carcinogenesis and for deciding the follow-up period after surgery for endometriosis. We are studying a larger number of cases of endometriosis to further validate our findings. We hypothesized that RET expression would be a good maker to assess the malignant potential of ovarian tumor. This study suggests that, in the future, RET may be useful for recognizing whether a patient has an ovarian cystadenoma or a potential malignant tumor before operation. We hope that the findings of our study will also lead to new targeted therapies for ovarian cancer.

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Research Article Open Access

Use of Localization and Activity of Thymidine Phosphorylase in Human Gynecological Tumors for Predicting Sensitivity to Pyrimidine Antimetabolite Therapy: An Observational Study

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Abstract

Background: Thymidine phosphorylase (dThdPase) is the rate-limiting enzyme in the conversion of 5'-deoxy-5-fluorouridine (5'-dFUrd), an intermediate metabolite of capecitabine (Xeloda®), to 5-fluorouracil (5-FU). We investigated the correlation between dThdPase activity and immunohistochemical staining in gynecological carcinoma and adjacent normal tissues. We hypothesize that the differential dThdPase activity between tumors and adjacent tissue is predictive of response to treatment with pyrimidine antimetabolites.

Methods: In 45 samples of carcinoma tissue and 35 of adjacent normal tissue from 45 patients, we measured dThdPase activity as well as immunoreactivity using an anti-dThdPase monoclonal antibody and macrophage and histiocyte-specific antibodies.

Results: dThdPase activity in tumor tissue was significantly higher than that in the corresponding adjacent normal tissue in all samples tested (12 uterine cervical, 19 endometrial, and 4 ovarian tumors). Anti-dThdPase immunopositivity was observed in the epithelial tumor cells of 76.9% of uterine cervical cancer samples, 60.0% of endometrial cancer samples and 63.6% of ovarian cancer samples. In stromal tissue, 84.6% of uterine cervical tumors (11/13), 90.0% of endometrial tumors (18/20), and 81.8% of ovarian tumors (9/11) were immunopositive for anti-dThdPase in interstitial cells (mainly macrophages). Macrophages were also strongly reactive in the stromal tissues of uterine cervical, endometrial, and ovarian cancers. The correlation between dThdPase activity and intensity of immunohistochemical staining of epithelial tumor cells with anti-dThdPase monoclonal antibody was statistically significant in endometrial carcinoma ($P = 0.008$) but borderline in uterine cervical tumors ($P = 0.077$). We found a good correlation between dThdPase activity and staining of epithelial tumor cells, particularly in the case of endometrial cancer.

Conclusions: We show that gynecological carcinomas show increased dThdPase activity, and this activity correlates with dThdPase staining of tumor epithelial cells. Thus, dThdPase staining of biopsy specimens could be useful in predicting the outcome of therapy with pyrimidine metabolites.

Keywords: Thymidine phosphorylase; Gynecological carcinoma; Pyrimidine antimetabolite therapy

Abbreviations: dThdPase: thymidine phosphorylase; 5'-dFUrd: 5'-deoxy-5-fluorouridine; 5-FU: 5-fluorouracil; HPLC: High Performance Liquid Chromatography; mAb: monoclonal antibody; ABC: Avidin-biotin conjugate

Introduction

Capecitabine (Xeloda®) and 5'-deoxy-5-fluorouridine (5'-dFUrd, Furtulon®) are masked compounds derived from 5-fluorouracil (5-FU) [1]. Thymidine phosphorylase (dThdPase) is the rate-limiting enzyme in the conversion of 5'-deoxy-5-fluorouridine (5'-dFUrd), an intermediate metabolite of capecitabine (Xeloda®), to 5-fluorouracil (5-FU). Although cytostatically inactive by themselves, they exert cytotoxic activity *in vivo* after being converted into 5-FU by the action of pyrimidine nucleoside phosphorylases [2-4], predominantly uridine phosphorylase in mouse and thymidine phosphorylase (dThdPase), reportedly identical to platelet-derived endothelial cell growth factor, in human tumors [5,6]. In addition, 5'-dFUrd is an active intermediate metabolite of capecitabine, which was approved for breast and colorectal cancer in the United States and the European Union. Capecitabine is actively catabolized by dThdPase in humans. From their mechanism of action, the antitumor activity of 5'-dFUrd and

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capecitabine is thought to depend upon the dThdPase activity of cells in tumor tissue, and a positive relationship between their antitumor effect and dThdPase activity has recently been reported [7]. Therefore, presumably, dThdPase values measured in human tumors can be utilized to optimize the efficacy of patients' treatment with 5'-dFUrd and capecitabine. To investigate that possibility, there have been many reports demonstrating dThdPase activity; however, dThdPase activity is dependent on the kind of gynecological cancer.

Analysis of dThdPase levels by high performance liquid chromatography (HPLC) has demonstrated that primary tumor tissues have higher dThdPase activity than adjacent normal tissue of the same organ [2,5,8-13]. Consequently, 5'-dFUrd is preferentially converted to 5-FU in primary tumor tissues after its administration [13]. Among gynecological tumors, however, the relationship between dThdPase activity in the tissues of uterine cervical, uterine endometrial, and ovarian cancers compared with those of adjacent normal tissues has not yet been established. In addition, there have been few reports on the histological localization of dThdPase in gynecological carcinoma tissues. Nevertheless, a report on immunohistochemical staining of uterine cervical carcinoma identified a correlation between survival and immunohistochemical localization of dThdPase [14]. The purpose of the present study is to clarify the relationship between the measured dThdPase activity and its immunohistochemical staining in gynecological cancers.

Material and Methods

Patients and samples

Between January 1993 and March 1994, 58 patients from 6 hospitals were enrolled into the present study (15 patients from Kinki University, Osaka; 13 patients from Hyogo Medical Center For Adults, Hyogo; 11 patients from Kurume University, Fukuoka; 9 patients from Jikei University, School of Medicine, Tokyo; 7 patients from Tohoku University, Miyagi; and 3 patients from Tokyo Metropolitan Komagome Hospital, Tokyo, Japan). The investigation was approved by the ethics review committees of all institutions, and all enrolled patients gave their informed consent that their tumor tissue, adjacent normal tissue, and lymph nodes could be used for analysis of dThdPase activity and immunohistochemical and histological examination. All specimens, which consisted of approximately lxlxl-cm samples of primary tumor tissue, adjacent normal tissue, and lymph nodes, were surgically resected and examined by the pathologists at the Department of Pathology, Jikei University School of Medicine. This pathology committee diagnosed histologically malignant tissue involvement in 45 of 58 patients, including 13 patients with uterine cervical carcinoma, 20 with endometrial carcinoma, and 12 with ovarian carcinoma. Histopathological diagnosis was carried out for both the tumor and adjacent normal tissues. Tissue from these 45 patients with malignancy were used for further analysis of dThdPase activity and immunohistochemical assay in the present study.

Reagents

5-FU was purchased from Kyowa Hakko Kogyo (Tokyo, Japan), and 5'-dFUrd was synthesized at F. Hoffmann-La Roche (Basel, Switzerland). Anti-dThdPase monoclonal antibody (mAb) 654-1 was provided by Nippon Roche Research Center (Kamakura, Japan). Anti-macrophage-CD68 mAb Kp-1 and anti-macrophage-CD68 mAb PG-M1 were purchased from DAKO Co. Ltd. (Glostrup, Denmark) and Vectastain Elite ABC Kit was purchased from Vector Laboratories, Inc. (Burlingame, CA, USA). Diaminobenzidine was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Assay of thymidine phosphorylase activity

Tissues were homogenized in 10 mM Tris-HCl buffer (pH 7.4) containing 15 mM NaCl, 1.5 mM MgCl, and 50 mM potassium phosphate. This solution was centrifuged at 105,000 × *g* for 90 min. The supernatant was dialyzed overnight against 20 mM potassium phosphate buffer (pH 7.4) containing 1 mM mercaptoethanol, and then used as a source of crude dThdPase. The protein concentration was determined by the method of Lowry et al. [15]. All procedures were carried out at 4°C. The reaction mixture (120 µl) for the enzyme activity assay contained 183 mM potassium phosphate buffer (pH 7.4), 10 mM 5'-dFUrd, and the crude enzyme from human tissue. The reaction was carried out at 37°C for 60 min and then terminated by adding 360 µl methanol. The precipitate was removed by centrifugation, and 100 µl supernatant was mixed with 20 µM 5-chlorouracil as the internal standard and applied to a HPLC column (ERC-ODS-1171). The 5-FU was eluted with 50 mM sodium phosphate buffer (pH 6.8) containing 5 mM 1-decanesulfonic acid:methanol (85:15, v/v) and measured with a UV monitor at 280 nm.

Immunohistochemistry

Tissues taken from the primary tumor lesion, adjacent normal tissue, and lymph nodes were used. Tumors included cancers of the uterine cervix, uterine endometrium, and ovary. Tissues were fixed with 10% formalin in saline for 24 to 72 h, dehydrated, and embedded in paraffin. Sections were cut at 4-µm thickness, placed on slides, deparaffinized in xylene, rehydrated, stained immunohistochemically by using an avidin-biotin conjugate (ABC) system with diaminobenzidine/hydrogen peroxide as substrate, and counterstained with hematoxylin [16]. Slides were cleared and coverslipped for microscopic examination at 200× magnification. The sections were also incubated with primary mAbs [17] at dilutions of 1:4000 for anti-dThdPase, 1:200 for anti-macrophage-CD68 clone Kp-1, and 1:1000 for anti-macrophage-CD68 clone PG-M1, which gave optimal intensity of specific staining with minimal nonspecific background reactivity [18]. The secondary or linking antibody was a biotinylated horse anti-mouse immunoglobulin (Vector Laboratories, Inc., Burlingame, CA). Immunohistochemical intensity was classified into four grades: (-), negative staining; (±), less than 10% positive cells; (+), from more than 10% to less than 50% positive cells; and (++), more than 50% positive cells. Both + and ++ staining were defined as positive, and both ± and - staining were defined as negative.

Statistical analysis

One and two-sample Wilcoxon tests were performed to detect differences in dThdPase activity between tumor and adjacent normal tissues. Logistic regressions were performed to detect correlations to dThdPase activity by factoring in the number of stained cells per field in addition to the intensity of immunohistochemical staining with anti-dThdPase mAb. All calculations were performed using Windows/SAS 6.12.

Results

dThdPase activity in gynecological tumor and normal tissues

Tumor samples obtained from the 45 patients enrolled in this study were analyzed for dThdPase activity, immunohistochemical staining, and histology for diagnosis of the tumor type. Additionally, 35 samples

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of normal tissue adjacent to the tumor were also analyzed for dThdPase activity. As shown in Table 1, dThdPase activity in tumor tissue was significantly higher than that in adjacent normal tissue in the 12 cases of uterine cervical cancer ($P = 0.001$, $P = 0.0002$, Wilcoxon one- and two-sample tests, respectively), 19 cases of endometrial carcinoma ($P = 0.0001$, $P = 0.0001$), and 4 cases of ovarian cancer ($P = 0.125$, $P = 0.025$)

		dThdPase activity[a]		Immunohistochemistry[b]		Histopathological
Organ	Case No.	Tumor tissue	Normal tissue	Epithelial cells	Stromal cells	diagnosis[c]
Uterine cervix	1	7.0	7.5	-	+	EC Ad Ca, HD
	2	172.5	NOS[d]	-	++	EC Ad Ca, PUD
	3	180.0	6.7	++	+	Sq Ca
	4	236.0	11.0	±	++	Sq Ca
	5	241.0	14.2	++	++	Sq Ca
	6	243.0	7.1	++	-	Sq Ca
	7	260.6	168.2	++	+	Sq Ca
	8	341.5	1.8	++	+	Sq Ca
	9	343.0	31.0	+	+	Sq Ca
	10	344.5	9.4	+	+	Sq Ca
	11	410.5	17.7	+	±	EC Ad Ca, PUD
	12	572.3	26.6	++	++	Sq Ca
	13	602.5	13.4	++	++	Sq Ca
Endometrium	1	15.1	43.9	-	+	EM Ad Ca
	2	20.6	28.0	-	-	EM Ad Ca
	3	41.5	6.2	-	+	EM Ad Ca
	4	50.8	8.4	+	++	EM Ad Ca
	5	56.8	7.5	±	±	EM Ad Ca
	6	70.0	1.3	-	+	EM Ad Ca
	7	81.6	11.1	-	+	EM Ad Ca
	8	81.8	8.3	-	++	Serous Ca
	9	111.2	73.2	+	++	EM Ad Ca
	10	117.6	9.2	+	++	EM Ad Ca
	11	120.0	NOS	+	+	EM Ad Ca
	12	129.0	6.5	+	++	EM Ad Ca
	13	135.0	5.1	-	++	EM Ad Ca
	14	161.4	8.3	+	++	EM Ad Ca
	15	187.0	24.4	+	++	EM Ad Ca
	16	192.5	59.5	+	+	EM Ad Ca
	17	200.0	10.6	++	++	EM Ad Ca
	18	203.0	18.7	+	+	EM Ad Ca
	19	229.0	28.6	+	++	Clear cell Ca
	20	339.0	12.4	+	++	Serous Ca
Ovary	1	11.5	NOS	-	+	Mucinous Ca
	2	12.3	NOS	-	+	Endomet Ca
	3	21.3	NOS	+	+	Clear cell Ca
	4	42.5	11.6	+	-	Mucinous Ca
	5	48.0	NOS	-	++	Serous cyst Ca
	6	61.7	0.0	+	++	Mixed epithel Ca
	7	73.0	NOS	+	+	Clear cell Ca
	8	75.0	NOS	++	+	Clear cell Ca
	9	101.0	14.8	+	-	Clear cell Ca
	10	143.0	NOS	NOS	NOS	Serous cyst Ca
	11	238.4	NOS	-	++	Serous cyst Ca
	12	12852.5	19.3	++	++	Serous cyst Ca

a) dThdPase activity is expressed as μg FU produced per mg protein per hour.
b) Immunohistochemistry is scored by the number of cells showing positive staining for anti-dThdPase mAb:
(-), negative staining; (±), weak and less than 10% positive cells; (+), 10% to 50% positive cells; (++), more than 50% positive cells.
c) EC Ad Ca, endocervical adenocarcinoma; HD, highly differentiated; EMOD, endometrioid carcinoma; PUD, poorly differentiated adenocarcinoma; Sq Ca, squamous cell carcinoma; EM Ad Ca, endometrial adenocarcinoma; Serous Ca, serous adenocarcinoma; Serous cyst Ca, serous cyst adenocarcinoma; Endomet Ca, endometrial adenocarcinoma; Clear cell Ca, clear cell carcinoma; Mucinous Ca, mucinous cyst adenocarcinoma; Mixed epithel Ca; mixed epithelial adenocarcinoma.
d) NOS, no specimen

Table 1: Activity of a pyrimidine nucleoside phosphorylase, dThdPase, immunohistochemistry in gynecological tumors and adjacent normal tissue.

for which normal tissue was available.

Immunohistochemistry with anti-dThdPase, anti-macrophage-CD68 clone Kp-1, and anti-macrophage-CD68 clone PG-M1 mAbs

We observed positive dThdPase immunostaining of epithelial cells in 76.9% of cervical tumors of the uterus (10/13 samples), 60.0% of endometrial tumors (12/20 samples), and 63.6% of ovarian tumors (7/11 samples), for a mean rate of 66.8% of gynecological cancers demonstrating dThdPase immunopositivity in epithelial cells. Interestingly, in stromal tissue, 84.6% of uterine cervical tumors (11/13), 90.0% of endometrial tumors (18/20), and 81.8% of ovarian tumors (9/11) were immunopositive for anti-dThdPase in interstitial cells (mainly macrophages). In immunohistochemical staining of normal epithelium and stromal tissue, weak reactivity to dThdPase was observed only in the nuclei of basal cells and the cytoplasm of superficial cells. In contrast, in the epithelium of uterine cervical carcinoma, immunoreactivity was present in both the primary carcinoma cells themselves and in the interstitial cells. In squamous cell carcinoma samples, both nuclei and cytoplasm were immunoreactive. The nucleus was stained in superficial carcinoma cells, while cytoplasmic staining was observed in both invasive and parabasal-layer cells (Figure 1). Cytoplasmic staining was often seen in the well-differentiated squamous cell carcinoma. In endocervical adenocarcinoma of the uterus, the highly differentiated type showed cytoplasmic staining of interstitial cells only (data not shown). In endometrial carcinoma, epithelial cells stained weakly with anti-dThdPase mAb (Figure 2). However, the staining was more intense in stromal cells than in epithelial tumor cells. Antimacrophage staining with CD68 and Kp-1 mAbs showed that anti-dThdPase staining in stromal cells was localized mainly to macrophages. In ovarian carcinoma, both serous and mucinous adenocarcinomas showed negative staining of epithelial tumor cells with anti-dThdPase mAb; however, the staining was positive for epithelial cells of clear cell adenocarcinoma (Figure 1). Some of the interstitial cells surrounding carcinoma cells showed stronger staining than the carcinoma cells themselves. Morphologically, the interstitial cells stained with anti-dThdPase mAb appeared to be macrophages or histiocytes in all tumors (Figure 1). They were also immunopositive for anti-macrophage mAb PG-M1 and/or anti-CD68 mAb Kp-1 in endometrial carcinoma, as shown in Figure 2. In the lymph nodes, premature lymphocytes, macrophages, and histiocytes showed strong positive staining, but the lymphocytes themselves were not immunopositive (data not shown).

Correlation between dThdPase activity and intensity of immunohistochemical staining

Table 2 shows the results of logistic regression analysis of dThdPase activity and the intensity of immunohistochemical staining with anti-dThdPase mAb. A significant correlation was only observed in endometrial carconoma, but not in uterine cervical and ovarian carcinoma.

Discussion

Increased levels of dThdPase have been reported in many malignant tumors [5,9,12]. Although there have been a several reports that investigated the dThdPase activity in cervical, endometrial and ovarian carcinoma [19-21], it remains to be determined. In this study of gynecological malignant tumors, we found that uterine cervical, endometrial, and ovarian carcinoma also demonstrated higher dThdPase activity than adjacent normal tissue. Several studies of dThdPase expression in cancer have been performed, because this enzyme is thought to activate pyrimidine antimetabolites [6,22]; however, only

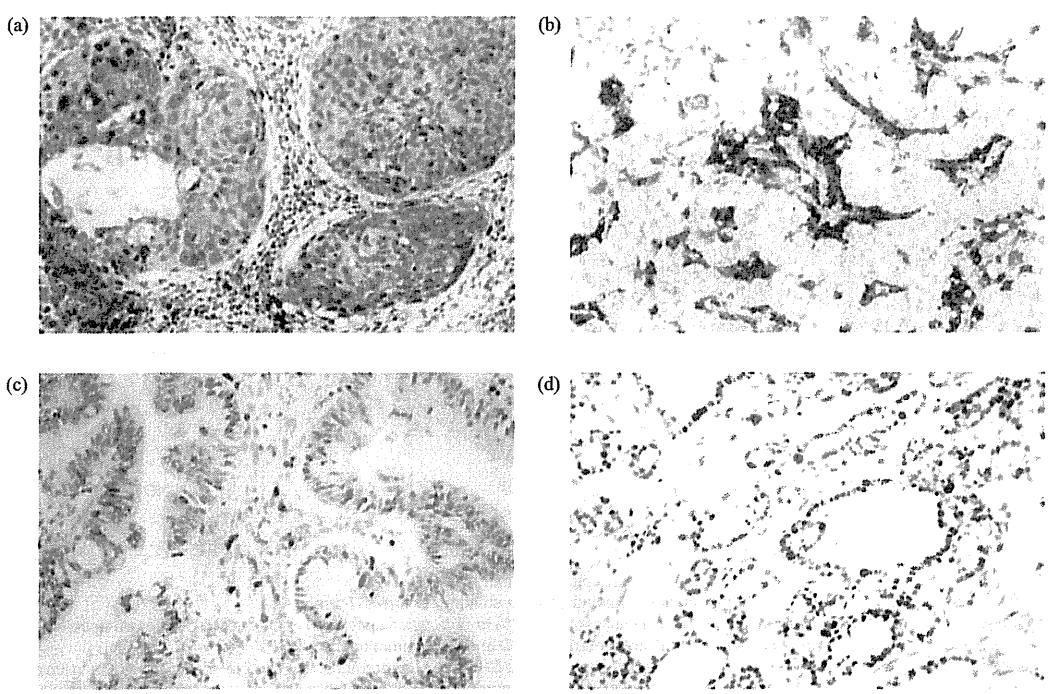

Figure 1: Immunohistochemical staining of squamous cell carcinoma with an anti-dThdPase mAb. (a) Uterine cervical carcinoma, (b) a serous papillary carcinoma of the ovary, (c) a mucinous adenocarcinoma of the ovary, and (d) a clear cell adenocarcinoma of the ovary.

a few reports have demonstrated the localization of dThdPase in tumor tissues [14,23,24]. In the present study, immunohistochemical staining with anti-dThdPase mAb revealed that dThdPase was strongly localized to epithelial tumor cells in squamous cell carcinoma of the uterine cervix. In endometrial carcinoma, dThdPase was found to be localized to epithelial tumor cells and, in stromal cells, mainly to macrophages. Serous and mucinous adenocarcinomas, both histologically serous types of ovarian cancer, showed no staining of carcinoma cells themselves. In contrast, in clear cell adenocarcinoma of the ovary, which constitutes less than 20% of ovarian cancer cases, dThdPase was localized to epithelial cells. Therefore, the relatively low dThdPase activity of ovarian cancer may be related to the absence of immunoreactivity in carcinoma cells.

Recently, some investigators have suggested that stromal dThdPase status may be a prognostic factor for survival [14,25,26], and some basic and clinical reports on dThdPase indicate that it is a predictive factor

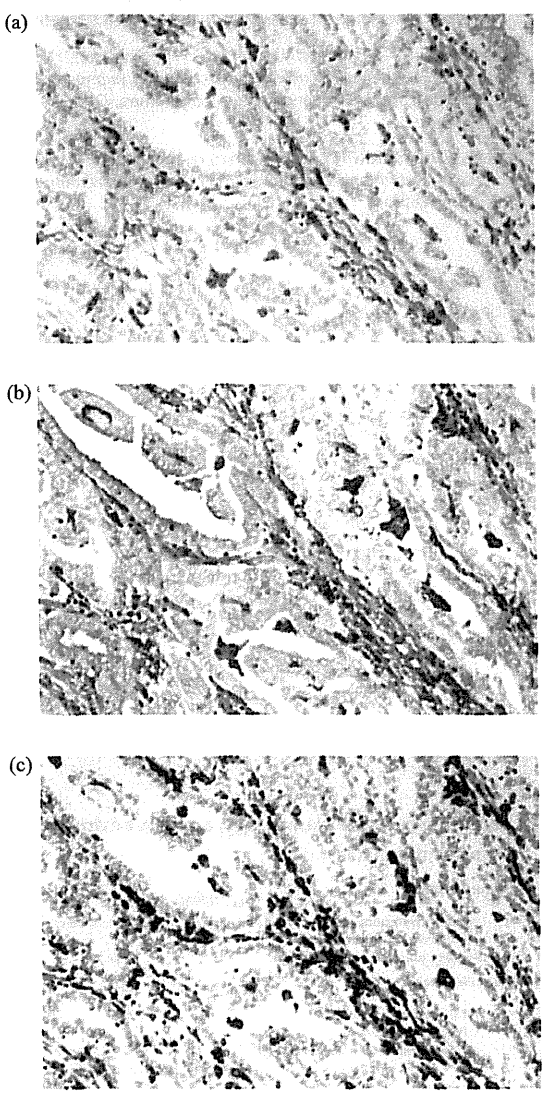

Figure 2: Immunohistochemical staining of an endometrial adenocarcinoma by the indirect method with ABC. (a) With anti-macrophage (PG-M1) mAb, (b) with anti-dThdPase mAb, and (c) with anti-CD68(Kp-1)mAb.

	Epithelial cells			Stromal cells		
	P-value	Odds ratio	(95% CI)	*P*-value	Odds ratio	(95% CI)
Uterine cervix	0.077	1.009	(0.999-1.019)	0.432	1.003	(0.996-1.010)
Endometrium	0.008	1.023	(1.006-1.041)	0.039	1.017	(1.001-1.034)
Ovary	0.185	1.005	(0.997-1.014)	0.308	1.011	(0.990-1.033)

Table 2: Results of logistic regression of dThdPase activity and intensity of immunohistochemical staining with anti-dThdPase mAb.

for 5'-dFUrd and capecitabine in the treatment of several carcinomas [25,27]. The results of logistic regression analyses of dThdPase activity and immunohistochemical staining intensity with anti-dThdPase mAb in the present study illustrate that dThdPase activity was well correlated with the intensity of staining in the epithelium in endometrial carcinoma and borderline in cervivcal carcinoma, but not with staining of stromal cells in cervical and ovarian carcinoma. Our resolution power was not sufficient to detect a correlation between dThdPase activity and stromal staining intensity. These results suggest that dThdPase activity reflects the intensity of immunohistochemical staining of epithelial tumor cells, as shown by their correlation, in endometrial carcinoma. The total immunohistochemical intensities did not significantly correlate with their enzymatic activities because of the lack of correlation between stromal cells and immunochemical staining in cervical carcinoma, and between both stromal and epithelial cells and immunochemical staining in ovarian carcinoma. Only a few reports that showed the good correlation between dThdPase activity and immunochemical staining in gynecologic cancer have been published [28,29], and it should be confirmed in other studies. In case of cervical carcinoma, since almost all squamous cell carcinomas showed very high dThdPase activity, the statistical correlation may have been obscured. The statistical correlation between dThdPase activity and intensity if immunohistochemistry was proven only for endometrial carcinomas, and that of uterine cervix was borderline. For ovarian carcinoma, data are sparse. dThdPase gene expression was proven to be significantly high in ovarian carcinoma [29] as shown in our study. Although good dThdPase activity and immunochemical staining were well correlated besed on the past reports [19], our study did not show the correlation. The number of cases may have been too small for sufficient statistical power.

Conclusion

We investigated the correlation between dThdPase activity and immunohistochemical staining in gynecological carcinoma and adjacent normal tissues. Our hypothesis is that the differential dThdPase activity between tumors and adjacent tissue will be predictive of response to treatment with pyrimidine antimetabolites. We show that gynecological carcinomas show increased dThdPase activity, and this activity correlates with dThdPase staining of tumor epithelial cells. Thus, dThdPase staining of biopsy specimens might be useful in predicting the outcome of therapy with pyrimidine metabolites.

Competing Interests

The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

Cancellation of in vitro fertilization treatment cycles predicts treatment outcome in female infertility patients aged 40 years or older

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Abstract
Purpose To investigate potential indicators of in vitro fertilization (IVF) treatment outcome for female infertility patients aged ≥ 40 years based on the clinical course.
Methods We retrospectively examined results of 111 female infertility patients aged ≥ 40 years undergoing IVF treatment. We investigated the relationship between treatment cycle cancellation and the final outcome of IVF treatment in female infertility patients aged ≥ 40 years.
Results A total of 44 pregnancies were achieved. Overall pregnancy rate per initiated treatment cycle was 12.1%, and 24 spontaneous abortions occurred (54.5%). No woman aged ≥ 45 years achieved pregnancy. No patients conceived after 10 treatment cycles while 42 (11.5%) oocyte pick-up cycles and 120 (33.0%) embryo transfer cycles were canceled. Investigation of correlation with treatment cycle cancellation revealed that patients who experienced embryo transfer cancellation had a high spontaneous abortion rate while only a few patients who experienced oocyte pick-up cancellation achieved pregnancy and even fewer achieved a successful outcome.
Conclusions Our study suggests that, in addition to patient age and number of treatment cycles, cancellation of treatment cycle also provides another useful indicator for pregnancy outcome.

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Keywords Cancellation · Embryo transfer · Female infertility patients aged 40 years or older · IVF · Oocyte pick up

Introduction

Many female infertility patients have conceived and achieved live births with assisted reproductive technology (ART); however, the outcomes of ART in patients aged ≥ 40 years are still unfavorable and the development of more effective treatment for these women is desired. In Japan in recent years, treatment cycles for female infertility patients aged ≥ 40 years are markedly increasing; it is not uncommon for female infertility patients to continue infertility treatment until the menopause. Some nations permit oocyte donation through legislation addressing assisted reproduction. In Israel, for example, national health insurance covers most infertility treatments, including in vitro fertilization and embryo transfer (IVF–ET), for the first two children. The age limit for performing IVF–ET with an infertile woman's own oocytes is 45 years [1].

In Japan, oocyte donation is not permitted according to the guidelines of the Japan Society of Obstetrics and Gynecology. Therefore, Japanese female infertility patients can only receive IVF–ET treatment using their own oocytes. Furthermore, Japanese women are traditionally expected to bear children [2, 3] with some Japanese infertility patients continuing to undergo infertility treatment because of parental pressure rather than their own desire [3]. Such social and traditional backgrounds concerning reproduction produce considerable stress for Japanese female infertility patients aged ≥ 40 years. The outcome of infertility treatment should be explained to such patients beforehand. With regard to factors influencing the outcome of IVF–ET, Tsafrir

et al. [1] noted in their retrospective analysis of 1,217 IVF cycles in women aged ≥ 40 years that none of 10 patients treated after 45 years of age delivered successfully. Klimpstein et al. [4] reported that the highest cycle day 3 follicle stimulating hormone (CD3 FSH) level in a 43-year-old woman achieving pregnancy was 18.0 mIU/mL and the highest CD3 FSH in a 40-year-old woman delivering a live child was 15.4 mIU/mL in their review of 2,705 cycles. Age and CD3 FSH level may thus provide useful indicators for treatment cut-off; however, an indicator for treatment cut-off based on the clinical course would be a more useful and more readily identified parameter for Japanese female infertility patients aged ≥ 40 years. The purpose of the present study was to summarize our experience regarding infertility treatment for these women and to determine an indicator for treatment cut-off based on the clinical course.

Materials and methods

The study included patient data from all infertile women aged ≥ 40 years at the time of IVF–ET treatment from 2004 to 2008 at Jikei University Hospital, Fuji Central Hospital and Kusuhara Ladies Clinic in Japan. A total of 111 women underwent 364 initiated cycles. Age distribution at the time of IVF–ET treatment is presented in Table 1. The protocol for ovarian stimulation was determined based on ovarian reserves, estimated by either CD3 FSH or previous ovarian response. The long, desensitization protocol using gonadotropin-releasing hormone (GnRH) agonist was conducted in patients estimated to have good ovarian reserve. The short protocol using a GnRH antagonist was conducted in patients estimated to have poor ovarian reserve. The clomiphene citrate or natural protocol was applied to the women in whom zero or one follicle developed in a previous long protocol, short protocol, or GnRH antagonist protocol.

Conventional IVF or intracytoplasmic sperm injection (ICSI) was performed depending on sperm condition or fertilization results in previous treatment cycles. Embryo transfer was performed on day 3. Cryopreserved-thawed embryo transfer was performed in cycles with a high risk of ovarian hyperstimulation syndrome or suboptimal endometrium.

Women were tested for urinary human chronic gonadotropin (HCG) about 14 days after embryo transfer. If this pregnancy test was positive, a clinical pregnancy was diagnosed based on the presence of an intrauterine gestational sac on transvaginal ultrasound. When pregnancy was diagnosed, luteal support was continued until 8 weeks' gestation.

Initial analysis included the results of all 364 initiated treatment cycles regarding rates of pregnancy, spontaneous abortion, and cancellation. Subsequent analyses comprised comparisons of: (1) outcome by age; (2) outcome by number of treatment cycles; and (3) the relationship of outcomes with cancellation cycles and patient background to cancellation cycles, respectively.

Statistical analysis

Student's t test was applied for continuous variables, and the chi-squared (χ^2) test was used for binary variables. A P value <0.05 was considered statistically significant.

Results

Demographic data (Table 2)

During the study period, 364 treatment cycles including oocyte pick-up cancellation cycles and embryo transfer cancellation cycles were initiated in 111 female infertility patients aged ≥ 40 years. A total of 42 (11.5%) oocyte pick-up cycles were canceled primarily because of poor ovarian response, while 120 (33.0%) embryo transfer cycles were canceled because of non-retrieval of an oocyte, no fertilized ovum, or nonviable embryo quality. A total of 44 pregnancies were achieved. Overall pregnancy rate per initiated treatment cycles was 12.1%. Pregnancy rate per treatment cycle, excluding oocyte pick-up or embryo transfer cancellation, was 21.8%; 24 of 44 cycles in which a pregnancy was achieved terminated in a spontaneous abortion (54.5%). The cause of infertility was examined in

Table 1 Age distribution of 364 IVF cycles in 111 patients

Age (years)	Total no. of cycles	Group A (18 patients 141 cycles)	Group B (93 patients 223 cycles)	Group C (57 patients 261 cycles)	Group D (54 patients 103 cycles)
40	89	16	73	62	27
41	77	18	59	49	28
42	60	23	37	39	21
43	50	25	25	38	12
44	52	34	18	41	11
45-	36	25	11	32	4

Table 2 Outcome of 364 IVF cycles in 111 patients aged ≥ 40 years

Parameter	
No. of pregnant patients	33
No. of pregnancy cycle	44
No. of spontaneous abortion cycles	24
Pregnancy rate per initiated cycle (%)	12.1
Delivery rate per initiated cycle (%)	5.5
Spontaneous abortion rate (%)	54.5
No. of oocyte pick-up cancellation cycles	42
Cancellation rate of oocyte pick-up per initiated cycle (%)	11.5
No. of oocyte embryo transfer cancellation cycles	120
Cancellation rate of embryo transfer per initiated cycles (%)	33.0

all 111 patients. As shown in Fig. 1, the majority of cases were determined to be unexplained infertility (55%). The clomiphene citrate protocol was most frequently used in controlled ovarian hyperstimulation (Fig. 2). There were no significant differences in the pregnancy rates between the five protocols.

Outcome of IVF treatment cycles according to age (Table 3)

Pregnancy rates averaged 12.9% between 40 and 44 years of age but dropped to 0% at ≥ 45 years. Rates of spontaneous abortion and cancellations for oocyte pick-up and embryo transfer increased considerably at ≥ 44 years.

Outcome of IVF treatment cycles according to cycle number (Table 4)

No patients conceived after ≥ 10 treatment cycles. Spontaneous abortion rates increased considerably after six treatment cycles. Combined cancellation rates for oocyte pick-up and embryo transfer increased to > 40% at ≥ 4 treatment cycles.

Relationship between outcome of IVF treatment cycles and cancellation of treatment cycle

Treatment cycles of patients aged ≥ 40 are often cancelled at oocyte pick-up or embryo transfer; such cancellations indicate an unfavorable outcome. We examined the relationship between cancellation of oocyte pick-up or embryo transfer and outcome of ART in the present patients. Initially, patients were classified into two groups according to whether they experienced oocyte pick-up cancellation. A total of 18 patients (141 cycles) who had oocyte pick-up canceled, primarily because of poor follicular response, were assigned to Group A. Group B comprised 93 patients (223 cycles) without oocyte pick-up cancellation. Clinical

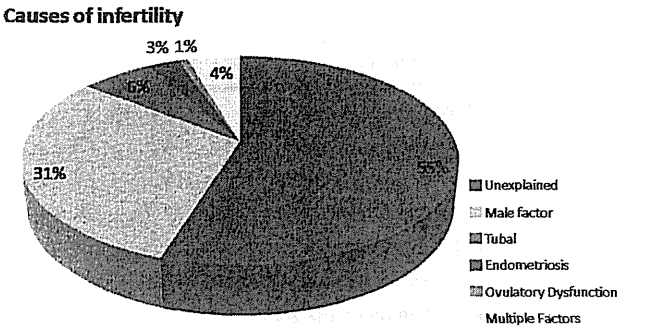

Fig. 1 The majority of cases were determined to be unexplained infertility (55%)

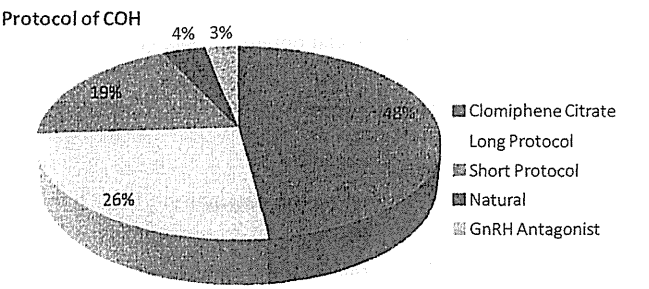

Fig. 2 The clomiphene citrate protocol was most frequently used in controlled ovarian hyperstimulation

Table 3 Outcome of 364 IVF cycles in 111 patients aged ≥ 40 years stratified by age

Age	40	41	42	43	44	45+
No. of treatment cycles	89	77	60	50	52	36
No. of oocyte cancellation cycles	7	5	8	3	11	8
No. of embryo transfer cancellation cycles	25	22	15	19	21	18
Cancellation rate (%) (OPU + ET)	36	35.1	38.3	44	61.5	72.2
No. of pregnancy cycle (%)	11	16	7	4	6	0
Pregnancy rate per initiated cycle (%)	12.4	20.8	11.7	8	11.5	0
Delivery rate per initiated cycle (%)	5.6	9.1	8.3	4	1.9	0
No. of spontaneous abortion cycles	6	9	2	2	5	–
Spontaneous abortion rate (%)	54.5	56.3	28.6	50	83.3	–

background (present age, age at marriage, age at onset of infertility treatment, duration of infertility, duration of infertility treatment prior to IVF, and cause of infertility) and ovarian reserves (CD3 FSH, human menopausal gonadotropin (hMG) ampoules (i.e., 75 IU), and number of oocytes retrieved) were compared. No significant differences were observed between the two groups with regard to clinical background (Table 5); however, Group A had significantly higher CD3 FSH and significantly lower oocyte retrieval (Table 6). These results indicate that the

Table 4 Outcome of 364 IVF cycles in 111 patients aged ≥ 40 years stratified by treatment cycle number

Treatment cycle number	1	2	3	4	5	6	7	8	9	10+
No. of treatment cycles	78	57	47	35	27	17	14	12	10	67
No. of oocyte cancellation cycles	4	5	3	6	7	2	1	3	1	10
No. of embryo transfer cancellation cycles	19	17	12	10	8	7	5	2	4	36
Cancellation rate (%) (OPU + ET)	29.5	38.6	31.9	44.7	55.6	52.9	42.9	41.7	50	68.7
No. of pregnancy cycle (%)	18	7	3	2	3	3	3	4	1	0
Pregnancy rate per initiated cycle (%)	23.1	12.3	6.4	5.7	11.1	17.6	21.4	33.3	10	0
Delivery rate per initiated cycle (%)	10.3	8.8	2.1	5.7	7.4	0	7.1	8.3	0	0
No. of spontaneous abortion cycles	10	2	2	0	1	3	2	3	1	0
Spontaneous abortion rate (%)	55.6	28.6	66.7	0	33.3	100	66.7	75	100	–

Table 5 Patient background differences regarding the cancellation of oocyte pick-up

	Group A ($n = 18$)	Group B ($n = 93$)	
Age (years)	42.5 ± 0.5	41.3 ± 0.2	n.s.
Age of marriage (years)	35.1 ± 1.4	34.5 ± 0.2	n.s.
Age at onset of infertility treatment (years)	39.5 ± 0.5	39 ± 0.3	n.s.
Duration of infertility (months)	23.3 ± 6.1	27.5 ± 3.5	n.s.
Duration of infertility treatment prior to IVF (months)	14.1 ± 2.7	12.1 ± 1.3	n.s.
CD3 FSH (mIU/ml)	16.5 ± 1.0	10.2 ± 0.4	$P < 0.001$*
Ampoules (i.e., 75 IU) of gonadotropin	14.6 ± 1.4	30.0 ± 1.0	$P < 0.001$*
No. of oocytes retrieved	1.9 ± 0.2	4.3 ± 0.2	$P < 0.001$*
Cumulative pregnancy rate per patient (%)	16.7	44.1	$P = 0.06$**
Pregnancy rate per initiated cycle (%) (without oocyte pick-up cancellation cycles)	3	18.4	$P < 0.001$**
Spontaneous abortion rate (%)	100	51.2	n.s.

* Student t test was used for statistical analysis

** The chi-squared test was used for statistical analysis

ovarian reserves of group A were significantly less than that of Group B. On the other hand, Group A had significantly lower hMG ampoules. This result depends on the fact that Group A contained a higher rate of the clomiphene citrate, or natural protocol cycles compared with that of Group B.

The cumulative pregnancy rate per patient for Group A was significantly lower than that of Group B (16.7 vs. 44.1%). The pregnancy rate per treatment cycle in which embryo transfer was conducted was significantly lower for Group A than Group B (3 vs. 18.4%). Moreover, all three pregnancies in Group A terminated in spontaneous abortion.

Subsequently, patients were classified into two groups according to whether they experienced embryo transfer cancellation (Table 6). A total of 57 patients (261 cycles) experiencing embryo transfer cancellation, mainly because of fertilization failure or lack of viable embryo, were assigned to Group C. Group D comprised 54 patients (103 cycles) without embryo transfer cancellation. The clinical backgrounds (present age, age at marriage, age at onset of infertility treatment, infertility duration, duration between onset of infertility treatment and onset of ART, and cause of infertility) and ovarian reserves (CD3 FSH, hMG ampoules, and number of oocytes retrieved) were compared. No significant differences were observed between the two groups with regard to clinical background; however, Group C had significantly higher CD3 FSH and hMG ampoules and significantly lower oocyte retrieval. These results revealed that the ovarian reserves of Group C were significantly lower than that of Group D. There was no significant difference between Groups C and D in the cumulative pregnancy rate per patient (40.4% vs. 38.9%) or in the pregnancy rate per treatment cycles in which embryo transfer was performed (22.8% vs. 20.8%); however, the spontaneous abortion rate of Group C (73.9%) was significantly higher than that of Group D (33.3%).

Table 6 Patient background differences in cancellation of embryo transfer

	Group C ($n = 57$)	Group D ($n = 54$)	
Age (years)	41.9 ± 0.3	41.3 ± 0.2	n.s.
Age of marriage (years)	34.1 ± 0.8	35.2 ± 0.5	n.s.
Age at onset of infertility treatment (years)	38.8 ± 0.6	39.4 ± 0.3	n.s.
Duration of infertility (months)	28.7 ± 5.0	24.8 ± 3.5	n.s.
Duration of infertility treatment prior to IVF (months)	12.7 ± 1.8	12.1 ± 1.5	n.s.
CD3 FSH (mIU/ml)	14.2 ± 0.6	8.7 ± 0.4	$P < 0.01$*
Ampoules (i.e., 75 IU) of gonadotropin	21.2 ± 1.2	31.1 ± 1.3	$P < 0.001$*
No. of oocytes retrieved	2.8 ± 0.2	5.4 ± 0.4	$P < 0.001$*
Cumulative pregnancy rate per patient (%)	40.4	38.9	n.s.
Pregnancy rate per initiated cycle (%) (without embryo transfer cancellation cycles)	22.8	20.8	n.s.
Spontaneous abortion rate (%)	73.9	33.3	$P < 0.05$**

* Student t test was used for statistical analysis

** The chi-squared test was used for statistical analysis

Discussion

In 2007, approximately 600 IVF institutions were registered with the Japan Society of Obstetrics and Gynecology and 160,000 treatment cycles (including conventional IVF fresh embryo transfer and ICSI, fresh embryo transfer or cryopreserved-thawed embryo transfer) were performed in Japan. The present status of ART reflects the high desirability of pregnancy in Japan which is due not only to the wishes of the patient but also family pressure as many Japanese still believe that motherhood is a wife's duty [2, 3]. Perhaps due to the historical background, the continuation of infertility treatment by Japanese female infertility patients may represent a sense of social duty as well as personal desire.

Oocyte donation is not permitted in Japan so infertile women must undergo treatment with their own oocytes; some women continue treatment until the menopause. In the present study, we summarized the treatment of female infertile patients aged ≥ 40 years and examined individual data to determine a cut-off point for infertility treatment.

Aggregate rates of pregnancy and delivery per initiated treatment cycle were 12.1% and 5.5%, respectively. In infertile women aged ≥ 40 years, Klipstein et al. [4] reported a pregnancy rate per initiated treatment cycle of 17.3% in 2,705 initiated IVF treatment cycles, Las et al. [5] described a rate of 11.3% in 1,087 cycles, and Tsafrir et al. [1] described a rate of 7.3% in 1,217 cycles; all results were < 20%.

In the present study no pregnancies were achieved in women aged ≥ 45 at the time of treatment cycle onset. Klipstein et al. [4] reported that no pregnancies were achieved in women aged ≥ 46 and they concluded that pregnancies at ≥ 44 years of age are rare. Lass et al. [5] reported no pregnancies achieved in women aged ≥ 45 years at the time of treatment cycle onset, while Tsafrir et al. [1] recommended that women aged ≥ 44 years should not undergo IVF. The cut-off age for IVF treatment appears to be approximately 45 years.

In the present study, the highest treatment cycle in which a pregnancy was achieved was cycle 9; however, this pregnancy miscarried at 7 weeks' gestation. The highest treatment cycle in which an infant was delivered was cycle 8. Martin-Johnston et al. [6] reported that the likelihood of a successful outcome declines with each additional treatment cycle. They concluded that the most notable decrease in clinical pregnancy rates occurred after the third treatment cycle, and patients who fail to conceive after three IVF cycles should be counseled to consider other options. The correlation between treatment cycle number and treatment outcome should be evaluated further in Japan and the cut-off age should be determined excluding treatment options such as oocyte donation.

The percentage of initiated cycles for patients aged ≥ 45 years at treatment cycle onset was 9.9% (36 cycles). Furthermore, the percentage of non-cancelled cycles after 10 treatment cycles was 18.4% (67 cycles). These findings indicate that parameters for treatment cut-off are not well established.

Treatment cancellations for oocyte pick-up or embryo transfer often occur in women over 40 years of age undergoing IVF procedures. In the present study, 42 (11.5%) oocyte pick-up cycles and 120 (33.0%) embryo transfer cycles were canceled. Only a few studies have examined the correlation between cancellation of IVF treatment cycle and outcome. Using logistic regression analysis, Peñarrubia et al. [7] demonstrated that the history of an IVF/ICSI cancelled cycle due to poor follicular response in a standard

stimulation protocol is a better predictor of cancellation in subsequent treatment cycles than age or CD3 FSH. Our study found that in treatment cycles in which embryo transfer was conducted, the pregnancy rate for the 18 patients who experienced oocyte pick-up cancellation was only 3% (3 treatment cycles, 3 patients); furthermore, none of these pregnancies resulted in a live birth. Conversely, 23 of 57 patients who experienced embryo transfer cancellation became pregnant. For treatment cycles in which embryo transfer was performed, the pregnancy rate per treatment cycle was 22.8%; this percentage was not significantly different than that of patients who did not experience embryo transfer cancellation. However, the spontaneous abortion rate of the 23 patients who experienced embryo transfer cancellation was 73.9%, which was significantly higher than that of patients who did not experience canceled cycles (33.3%). The ovarian reserves of patients who experienced oocyte pick-up cancellation and embryo transfer cancellation were significantly lower than those of patients who did not experience either cancellation. The pregnancy rate per treatment cycle of patients who did not experience oocyte pick-up cancellation or embryo transfer cancellation was 21.3%; furthermore, the spontaneous abortion rate was 30%.

Lower ovarian reserves correlated with a poorer outcome for IVF treatment in women aged $\geq$ 40 years. Moreover, patients who experience embryo transfer cancellation have a higher spontaneous abortion rate if they achieve a pregnancy. In addition, few women who experience oocyte pick-up cancellation achieve a pregnancy, and fewer deliver a viable infant.

Klipstein et al. [4] reported that CD3 FSH values correlate with pregnancy, live birth, and pregnancy loss rates. Additionally, in their review of 2,705 cycles, they reported that the highest CD3 FSH level in a woman achieving a pregnancy was 18.0 mIU/mL, and that the highest CD3 FSH in a woman delivering a live child was 15.4 mIU/mL. The present study found that the highest CD3 FSH level in a woman achieving a pregnancy was 22.2 mIU/mL, and that the highest level in a woman delivering a live child was 17.6 mIU/mL. Therefore, IVF treatment may not be efficacious for women $\geq$ 40 years whose CD3 FSH is $>$ 20 mIU/mL. As CD3 FSH values fluctuate in each cycle, anti-Müllerian hormone may be a useful marker of ovarian reserves in women $\geq$ 40 years; thus, it would be of value regarding the decision of infertility treatment cut-off.

We evaluated potential indicators for treatment cut-off for women $\geq$ 40 years: age, number of treatment cycles, and cancellation of treatment cycle. The present findings suggest that cancellation of treatment cycles is as useful an indicator as age or the number of treatment cycle. However, further research is necessary with larger patient populations encompassing more parameters. Once established, realistic cut-off criteria for infertility treatment should be disseminated to Japanese society to help prevent social and family pressure. Moreover, consideration should be given to how, when and by whom counseling should be given regarding ending infertility treatment.

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New Terminology for Intrauterine Endometrial Samples: A Group Study by the Japanese Society of Clinical Cytology

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Key Words
Endometrial cytology · Intrauterine sampling · Descriptive reporting format · Sensitivity · Specificity · Group study

Abstract
Objective: To evaluate the sensitivity and specificity of endometrial cytology obtained by intrauterine sample using a descriptive reporting format for endometrial cytological diagnosis. ***Study Design:*** 10,152 consecutive endometrial scrapings obtained in 13 different Japanese hospitals were analyzed. Cytological results were classified as 'negative for malignancy', 'atypical endometrial cells' (ATEC), 'endometrial hyperplasia', 'atypical endometrial hyperplasia' or 'malignant tumor'. ATEC was subclassified as 'ATEC, of undetermined significance' (ATEC-US) and 'ATEC, cannot exclude atypical endometrial hyperplasia or more' (ATEC-A). Cytological results were compared with the histological diagnosis as a gold standard. When the cytological result was 'negative for malignancy' and there was no subsequent histological examination, the case was considered a true negative when the endometrium was assessed as normal on transvaginal ultrasonography and there was no abnormal uterine bleeding. ***Results:*** 1,083 cases in which histology was not performed, 557 cases of 'unsatisfactory specimen' and 76 cases of ATEC-US were excluded. In the remaining 8,436 cases, the sensitivity and specificity, positive predictive value and negative predictive value for detecting atypical endometrial hyperplasia or malignant tumors were 79.0 and 99.7, 92.9 and 98.9%, respectively. ***Conclusion:*** The current diagnostic standards for endometrial cytology in Japan were established. Specificity is satisfactory for excluding cancer or precancerous diseases.

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Introduction

In Japan, endometrial cytology by intrauterine sampling is one of the most common tests for evaluating the status of the endometrium [1]. This method is usually applied for the initial pathological evaluation and often for endometrial cancer screening tests, together with endocervical cytology. However, up to now, its precise sensitivity and specificity have not been evaluated. Usually, conventional smears are used in a screening setting and liquid-based preparations are limited to experimental usage. For reporting endometrial cytology, three categories, 'negative', 'suspicious', and 'positive', are usually applied. While 'positive' is used when a sample is assessed cytologically as malignant and 'negative' as benign, the definition of 'suspicious' is ambiguous. 'Suspicious' is usually applied when a sample is assessed as endometrial hyperplasia with or without cellular atypia; however, since it indicates a pathological condition that is neither entirely benign nor entirely malignant, it has no clinical usefulness and just serves to categorize the sample in the reporting process. We felt that this terminology could be adapted to the spectrum of conditions showing some degree of cytological atypia of the endometrial mucosa. To overcome the problems caused by a clinically unsatisfactory 'suspicious' report, we previously proposed the concept of an endometrial reporting format [1, 2]. In this study, we developed a new descriptive reporting format for endometrial cytology for actual clinical usage, which enabled a study group to evaluate the sensitivity and specificity of endometrial cytology in Japan on behalf of the Japanese Society of Clinical Cytology (JSCC). The goal of this study was to establish the feasibility of endometrial cytology in Japan.

Table 1. Descriptive reporting format for endometrial cytology for the JSCC group study

1 *Specimen type* Conventional method, liquid-based method
2 *Specimen adequacy* Satisfactory, unsatisfactory (rejected specimen, fully evaluated, unsatisfactory specimen)
3 *Result* Negative for malignancy Endometrium in proliferative phase, in secretory phase, in menstrual phase, atrophic endometrium, benign reactive change (IUD, TAM, etc.), endometrial polyp, simple endometrial hyperplasia ATEC: Atypical endometrial cells (ATEC-US or ATEC-A must be selected) ATEC-US: Atypical endometrial cells, of undetermined significance ATEC-A: Atypical endometrial cells, cannot exclude atypical endometrial hyperplasia, etc. Endometrial hyperplasia Complex endometrial hyperplasia Atypical endometrial hyperplasia Atypical endometrial hyperplasia, endometrial adenocarcinoma in situ, atypical polypoid adenomyoma Malignant tumor Endometrioid adenocarcinoma (G1, G2, G3, squamous differentiation), serous adenocarcinoma, clear cell adenocarcinoma, mucinous adenocarcinoma, squamous cell carcinoma, mixed carcinoma, undifferentiated carcinoma, mesenchymal tumors, endometrial stromal sarcoma, leiomyosarcoma, carcinosarcoma, homologous type, heterologous type, other malignant tumors, extrauterine malignant tumors

IUD = Intrauterine device; TAM = tamoxifen.

Materials and Methods

During the 2-year period ending March 2009, 13 hospitals applied the descriptive reporting format for endometrial cytological diagnosis (table 1). Each specimen entering the study was first tested for adequacy and then classified. For evaluating endometrial cytology, five new categories were set, namely: (1) negative for malignancy (fig. 1, 2); (2) atypical endometrial cells (ATEC; fig. 3); (3) endometrial hyperplasia (fig. 4); (4) atypical endometrial hyperplasia (fig. 5), and (5) malignant tumor (fig. 6). ATEC includes 'atypical cells, of undetermined significance' (ATEC-US) and 'atypical endometrial cells, cannot exclude atypical endometrial hyperplasia or more' (ATEC-A). ATEC-US is selected when atypical endometrial cells are observed but their significance cannot be determined for some reason, possibly inflammatory, metaplastic, iatrogenic or any other changes causing cytomorphological alterations. ATEC-A is selected when the possibility of atypical endometrial hyperplasia or malignant tumor is not excluded mainly because of the limited number of atypical cells in the absence of inflammation, metaplastic changes or iatrogenic influences. While a more detailed cytological diagnosis can be selected for cases of 'negative for malignancy', 'endometrial hyperplasia', 'atypical endometrial hyperplasia' or 'malignant tumor' with endometrial cytology, for cases evaluated as ATEC, either an ATEC-US or ATEC-A has to be selected without exception. When the cytological result is 'negative for malignancy', subsequent endometrial histological evaluation is not necessarily required. However, in cases of irregular endometrial ultrasonographic findings or accompanying abnormal uterine bleeding, endometrial biopsy or curettage must be considered even in 'negative for malignancy' cases. Because ATEC-US is expected to contain a spectrum ranging from benign endometrium to neoplastic changes, it is difficult to decide a triage method immediately. In this study, endometrial biopsy or repeat endometrial cytological

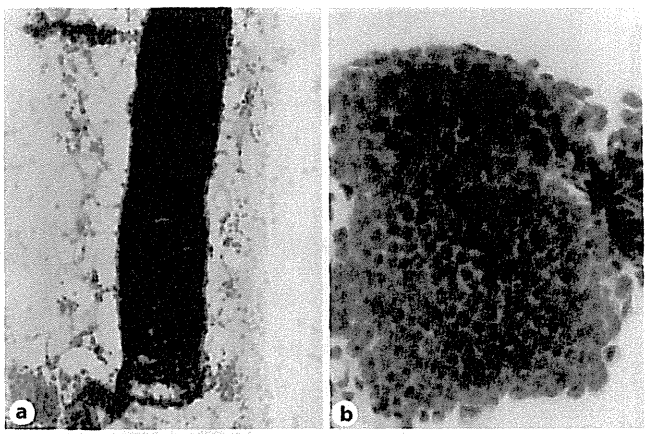

Fig. 1. Samples negative for malignancy. Cell clumps with tubed- and sheet-shaped pattern. These cells were obtained from endometrium in the proliferative phase. Usually, almost all epithelial cell clumps obtained from normal endometrium show a tubed- and sheet-shaped pattern. Stromal cells attached to the margins of tube-shaped endometrial glands are usually observed. In the proliferative phase, the width of the tube is approximately equal. **a** ×100. **b** ×400.

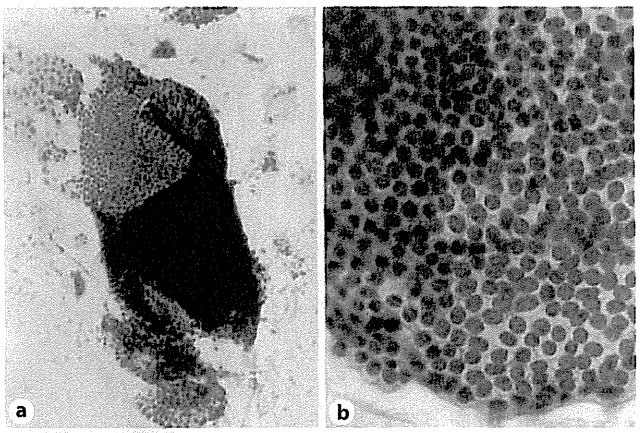

Fig. 2. Samples negative for malignancy. These cells were obtained from endometrium in the secretory phase. Cell clumps with tubed- and sheet-shaped pattern are dominant as in the proliferative phase endometrium. In the secretory phase, the maximum diameter of width of the tube is often more than twice the minimum diameter. In the mid-secretory phase a honeycomb pattern with well-defined and clear cytoplasm is observed. **a** ×100. **b** ×400.

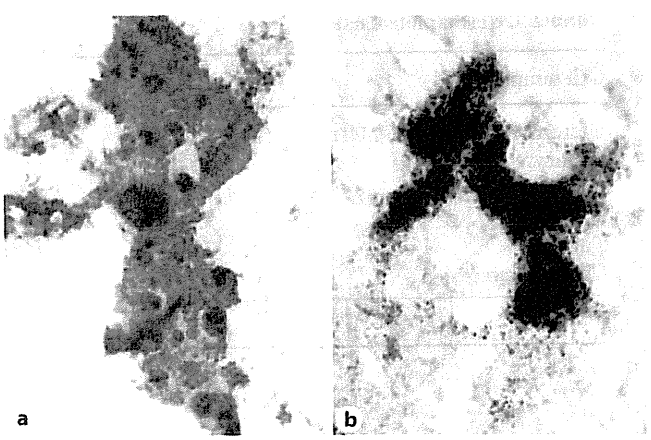

Fig. 3. ATEC samples. **a** ATEC-US. Cell clumps with irregular protrusions composed of metaplastic cells. This case was histologically diagnosed as benign endometrium with abnormal hormonal influence by endometrial tissue sample. Usually, when many cell clumps composed of metaplastic cells are observed, definite evaluation is difficult. In addition, this case being a dry specimen made it difficult to make precise cellular findings. ×200. **b** ATEC-A. Cell clumps with irregular protrusions or dilated and branched patterns exist. Because of the inflammatory background, precise cellular evaluation is difficult. In this case, in spite of obscured findings, atypical endometrial hyperplasia or endometrioid adenocarcinoma was not excluded because cellular atypia was strongly suspected. The histological diagnosis was atypical endometrial hyperplasia. ×200.

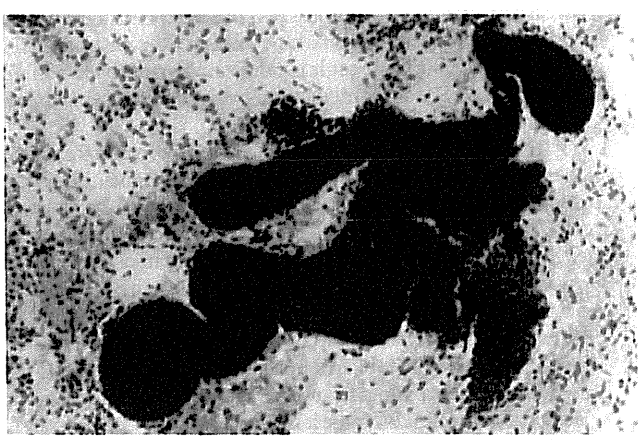

Fig. 4. Endometrial hyperplasia sample. A cell clump with a dilated and branched pattern. Because this cell clump is composed of endometrial glands, stromal cells attached to the margins of tube-shaped endometrial glands are also observed, as with endometrium of the proliferative and secretory phases. The cells composing these cell clusters resemble those of proliferative endometrium. ×400.