counterstained using Meyer hematoxylin followed by dehydration and coverslip mounting. Negative controls were performed by replacing the primary antibody with 1% bovine serum albumin.

A semi-quantitative scoring criterion was used to evaluate the IHC staining. A staining index valued from 0–5 was obtain from the sum of scores of the percentage of positive cells (0: <20% positive cells; 1: 20–80%; 2: >80%) and staining intensity (0: negative staining; 1: weak; 2: moderate; 3: strong). All slides were evaluated blindly for immunostaining without any knowledge of the clinical or pathological data. Score 0–1 was defined as low (including negative expression), score 2 as moderate, and >=3 as high expression of Cyr61.

Detection of Cyr61 expression by RT-PCR

To evaluate Cyr61 expression at the RNA level, 8 randomly-selected endometrial carcinoma tissues (all included in the cohort for immunohistochemisry) previously preserved were retrieved. Total RNA was isolated using Trizol reagent (Invitrogen, USA) according to the manufacturer's instruction. Two micrograms of RNA was used for complementary DNA (cDNA) synthesis employing avian reverse transcriptase and Oligo(dT) (ThermoScript™ RT-PCR System, Invitrogen, USA) and amplified by PCR using the following set of primers: forward: 5′-GGCTGCGGCTGCTGTAAGGTC-3′; reverse: 5′-GTTCGGGGGATTTCTTGGTCT-3′. PCR product indicating Cyr61 mRNA should be 718 bp in length. cDNA templates were amplified using Taq polymerase (Invitrogen, USA) per 10 μl of PCR reaction. 94° C for 2 min for initial melting was followed by 30 cycles of 94 °C for 1 min, 59°C for 1 min and 72°C for 1 min; 10 min at 72°C was used for final extension following cycling. In each PCR reaction buffer, the house-keeping gene GAPDH was simultaneously amplified using the following primers:5′-TGCCGTCTAGAAAAACCTGC-3′ (sense) and 5′-ACCCTGTTGCTGTAGCCAAA-3′(abtisense).

Statistical analysis

Statistical analysis was performed using the Statview 5.0 statistical software. The analysis of the correlation between Cyr61 expression and clinicopathological variables was performed by chi-square test or Fisher's exact test for categorical variables. A p value less than 0.05 was considered significant.

Results

Cyr61 expression in endometrioid adenocarcinoma

To confirm whether Cyr61 expression evaluated by immunohistochemistry was largely paralleled at the mRNA level, cDNAs from 8 endometrioid adenocarcinomas were amplified using Cyr61-specific primers. As shown in Fig. 1, T1–T4 had immunohistochemically high expression, while T5–T8 had low expression for Cyr61, indicating that down-regulation of Cyr61 in tumor tissues by immunohistochemistry was correlated with mRNA levels. We, therefore, further examined expression of Cyr61 in endometrioid adenocarcinoma by immunohistochemistry alone in this study.

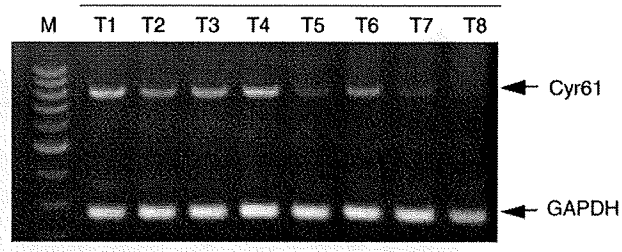

Fig. 1. Detection of Cyr61 expression by RT-PCR in endometrioid adenocarcinoma (M: DNA size marker; T1–T4: endometrioid adenocarcinoma with moderate-high expression of Cyr61 detected by IHC; T5–T8: endometrioid adenocarcinoma with immunohistochemically negative or low expression of Cyr61. Cyr61 mRNA transcripts were amplified using its specific primers for 30 cycles. GAPDH was amplified simultaneously and served as internal control.

A total of 92 endometrial carcinomas of endometrioid subtype were evaluated for Cyr61 expression. Cyr61 was mainly localized to the cytoplasm and representative results of immunohistochemistry were shown in Fig. 2. 45.6% (42/92) of tumors showed low Cyr61 expression, 31.5% (29/92) showed moderate, and 22.8% (21/92) showed high expression.

Correlation of Cyr61 expression with clinicopathological factors of endometrioid adenocarcinoma

To obtain a better understanding of the clinical significance of Cyr61 expression in endometrioid adenocarcinoma, we correlated its expression with a series of clinicopathological factors. As shown in Table 1, Cyr61 expression was not associated with any of the clinicopathological factors examined, including age (p=0.78), FIGO staging (p=0.79), architectural grade (p=0.85), nuclear grade (p=0.14), myometrial invasion (p=0.32), cervical invasion (p=0.24), lymphvascular space invasion (p=0.43), ovarian metastasis (p=0.72) and lymph node metastasis (p=0.57) in this cohort.

Prognostic impact of the Cyr61 expression in patients with endometrioid adenocarcinoma

Fig. 3 shows the survival of endometrial cancer patients according to Cyr61 expression. The estimated 5-year survival rate was 94.2% for patients with low/moderate expression of Cyr61 (n=71), 80.1% for those with high expression of Cyr61 (n=21). There was statistically significant difference of survival between low/moderate Cyr61 expression group and high Cyr61 expression group (p=0.03).

Univariate and multivariate survival analysis for patients with endometrioid adenocarcinoma (Table 2, Fig. 4)

Since Cyr61 expression was shown to have significant impact on the survival of patients with endometrioid adenocarcinoma, we included Cyr61 expression in the survival analysis. The univariate analysis revealed that the NG (p=0.03), LVSI (p=0.0013), LNM (p<0.0001), and Cyr61 expression (p=0.03) were shown to be

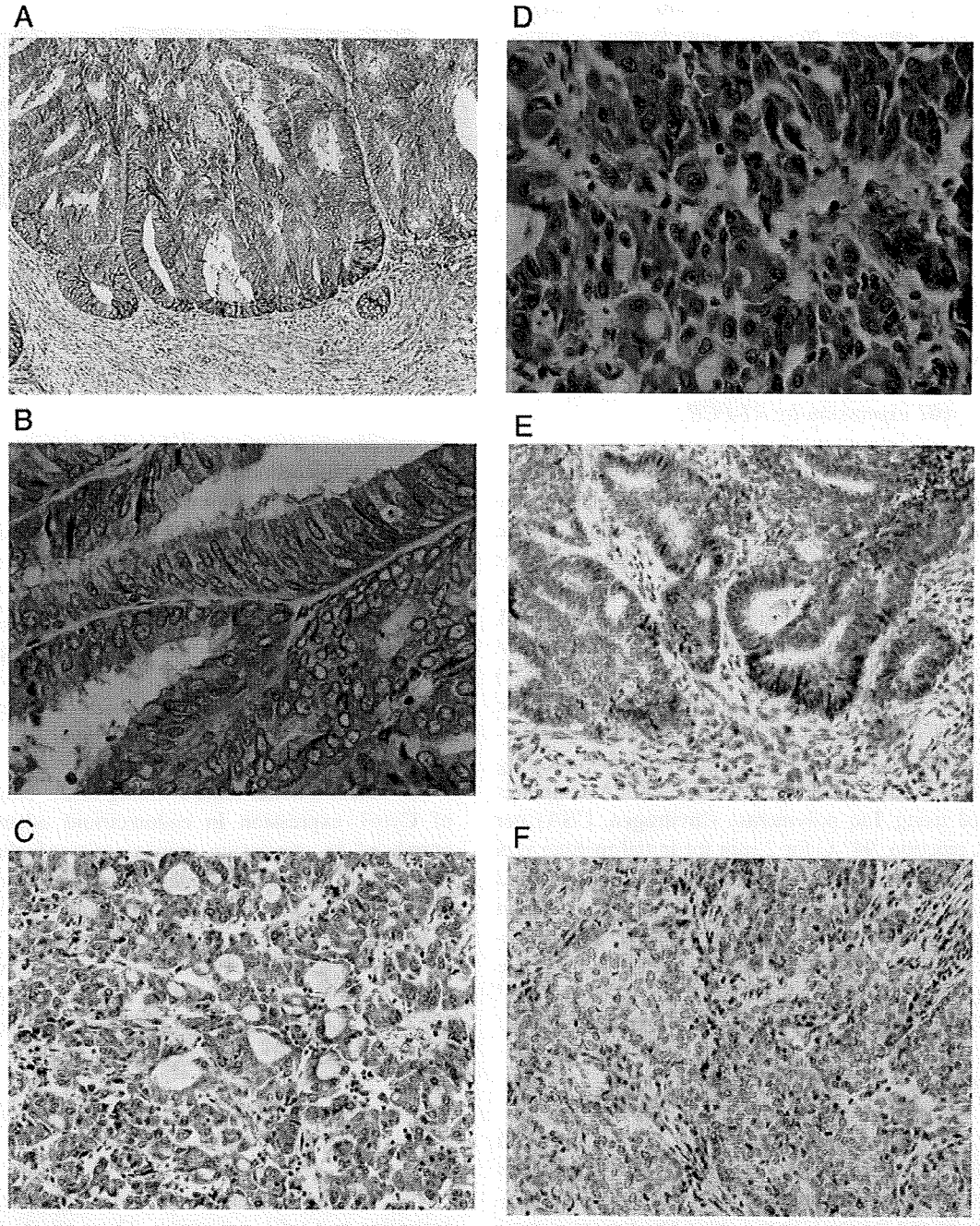

Fig. 2. Cyr61 expression detected by immunohistochemistry in endometrioid adenocarcinoma Cytoplasmic staining of Cyr61 in endometrioid adenocarcinoma tissues. A–D. High expression of Cyr61 in histologic grade 1 (A; ×100, B; ×400) and grade 3 (C; ×100, D; ×400) tumor. E and F. Loss of expression in grade 1 (E; ×100) and grade 3 (F; ×100) tumor.

related to poor survival. Age ($p=0.07$), AG ($p=0.44$), depth of myometrial invasion ($p=0.14$), cervical invasion ($p=0.13$), ovarian metastasis ($p=0.44$) were not related to poor survival in this cohort. Multivariate analysis revealed that LNM ($p=0.003$) and Cyr61 expression ($p=0.02$) were independent prognostic factors. Survival of patients could be stratified into three groups by combination of LNM and Cyr61 expression with an estimated 5-year survival rate of 96.5% for negative LNM irrespective of Cyr61 expression ($n=79$, group A), 85.7% for positive LNM with low/moderate expression of Cyr61 ($n=9$, group B), and 0% for positive LNM with high expression of Cyr61 ($n=4$, group C). The difference of survival rate between group B vs group C or group A vs group C was statistically significant ($p=0.008$ for group B vs group C, $p<0.0001$ for group A vs group C , respectively). There was no statistically significant difference of survival rate between group A and group B ($p=0.18$).

Discussion

Cyr61 was originally proposed to be a positive regulator of cell growth based on early observations. As a well-established pro-angiogenic factor, Cyr61 was found to be overexpressed

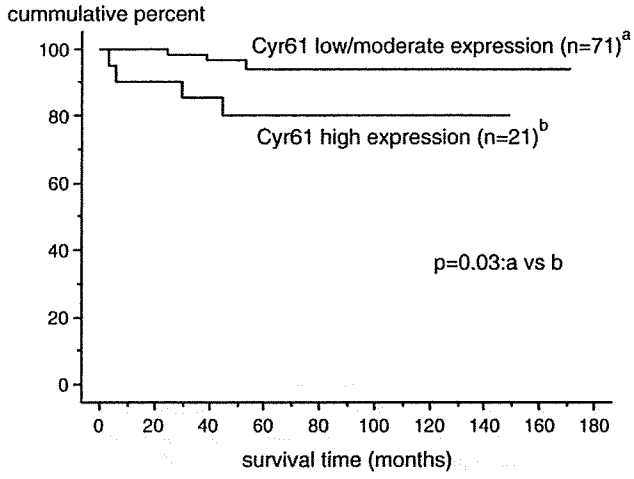

Fig. 3. Survival of patients with endometrioid adenocarcinoma by Cyr61 expression.

and associated with progression to more advanced stage and formation of larger tumors in breast cancer and breast cancer cell lines [5,6]. Similarly, transfection of Cyr61 into gastric adenocarcinoma cells results in formation of larger and more vascularized tumors [7]. Cyr61 is also expressed at high levels and correlated with worse prognosis in glioblastoma [15]. However, Cyr61 has been found in low levels in prostate cancer and thyroid carcinoma [10,11], and to be a cell growth suppressor in non-small-cell lung cancer [8,9].

Only two previous studies have been reported on Cyr61 expression in endometrial cancer. In the first study, Chien et al reported an inverse relationship between Cyr61 expression and growth of endometrial cancer cells. Most of the data presented in that study were obtained after examination of cancer cell lines In the portion of their study that did examine endometrial cancers, the authors compared Cyr61 mRNA expression in eight tumor samples with eight randomly selected normal tissue samples and the expression of Cyr61 in the control samples was highly variable [12]. On the contrary, MacLaughlan et al recently reported that Cyr61 is expressed in endometrial hyperplasia and in most adenocarcinoma samples by immunohistochemistry and western blot analysis [13]. In two previous studies, Cyr61 expression was examined in limited number of endometrial cancer samples (eight in Chien et al and fifteen in MacLaughlan et al). Therefore, this is the first report on the expression of Cyr61 in a large cohort of endometrial cancer tissues as far as we know. Our result seems consistent with those described by Chien et al and MacLaughlan et al. [12,13], because we found that some tumors expressed Cyr61, while some did not. Taken together, we can conclude that down-regulation of Cyr61 expression might contribute to tumorigenesis and/or the biological property of some endometrioid endometrial cancer cells. High expression of Cyr61 might also play a role in the progression and/or biological behavior of endometrial cancer cells.

To clarify the clinical significance of high expression of Cyr61 in endometrial carcinoma of endometrioid subtype, we further correlated Cyr61 expression with clinicopathological variables. Cyr61 expression was not related to any clinicopathological factors examined in this study (age, FIGO stage, AG, NG, myometrial invasion, CI, LVSI, ovarian metastasis, and LNM). However, MacLaughlan et al [13] described that Cyr61 expression appears to be regulated through a G-protein-coupled receptor, GPR30, which was recently shown to be a marker of poor prognosis in endometrial cancer [16]. They raised the possibility that poor prognosis in endometrial cancer may be mediated, in part, through Cyr61. In addition, since we found that high expression of Cyr61 was related to poor survival of patients with endometrioid adenocarcinoma (Fig. 3), we performed multivariate analysis on prognostic factors. We found that Cyr61 was an independent prognostic factor for patients with endometrioid adenocarcinoma as well as LNM (Table 2) and survival of patients with endometrioid adenocarcinoma could be stratified by the combination of Cyr61 expression and LNM (Fig. 4). Therefore, Cyr61 expression might be a new prognosticator for node-positive patients with endometrial cancer of endometrioid subtype. Although we do not know the exact reason why Cyr61 showed prognostic significance for node-positive patients with endometrioid adenocarcinoma, we speculate one possibility that high expression of Cyr61 may relate to the resistance to adjuvant therapy. We have given adjuvant chemotherapy for node-positive patients after extensive surgery [14]. Thus, Cyr61 may confer resistance to chemotherapeutic drugs in endometrial cancer cells. Indeed, Cyr61 has been reported to be related to resistance to carboplatin in ovarian cancer cells [17], and resistance to paclitaxel in breast cancer cells [18]. Possible mechanism of chemoresistance by Cyr61 might be not due to suppression of apoptosis, because overexpression of Cyr61 has been reported to induce apoptosis in endometrial cancer cells [12]. One possibility of chemoresistance by Cyr61 might be due to growth suppression of endometrial cancer cells by Cyr61, since cell proliferation is considered to be an important factor of chemoresponse in other cell types [19,20]. We, therefore, speculate that low-proliferative cells overexpressing Cyr61 do not respond well to cytotoxic drugs and may result in high recurrence rate and poor survival of node-positive patients with high Cyr61 expression who received adjuvant chemotherapy. We need to further investigate the functional role of Cyr61 in the mechanism of chemoresistance in endometrial cancer cells.

Table 2
Multivariate analysis on the prognostic factors for endometrioid adenocarcinoma

Univariate		Multivariate		
Clinicopathologic factor	*p* value	Risk ratio	95%CI	*p* value
Age	0.07	–	–	NS
Architectual grade	0.44	–	–	NS
Nuclear grade	0.03	–	–	NS
Lymph-vascular space invasion	0.0013	–	–	NS
Myometrial invasion	0.14	–	–	NS
Cervical invasion	0.13	–	–	NS
Ovarian metastasis	0.44	–	–	NS
lymphnode metastasis	<0.0001	22.7	4.2–125.0	0.0003
Cyr61 expression	0.03	6.0	1.3–28.6	0.02

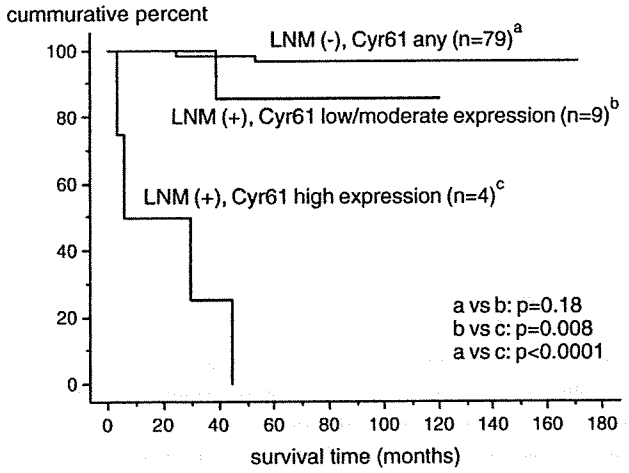

Fig. 4. Survival of patients with endometrioid adenocarcinoma by combination of Cyr61 expression and lymph node metastasis.

In summary, Cyr61 was *bona-fide* expressed in certain endometrioid adenocarcinoma tissues and high expression of Cyr61 was related to poor survival of patients with endometrioid adenocarcinoma. Cyr61 might be a new molecular marker to predict survival of node-positive patients with endometrioid adenocarcinoma. Further studies will be needed to establish the biological significance of Cyr61 expression and to examine the possibility as a therapeutic target in endometrial cancer cells.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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A 14-Year-Old Female Patient With FIGO Stage IB Endometrial Carcinoma

A Case Report

Takashi Mitamura, MD, Hidemichi Watari, PhD,* Yukiharu Todo, PhD,* Takayuki Koshida, MD,† and Noriaki Sakuragi, PhD**

Introduction: This is the second report on the conservative treatment of endometrial carcinoma in a female patient younger than fifteen years. Case A, a 14-year-old teenager, presented with menorrhagia. An endometrial biopsy revealed grade 2 endometrioid adenocarcinoma. The estrogen receptor and progesterone receptor were strongly positive in 60% and 90% of the tumor, respectively. Although she was administered medroxyprogesterone acetate for a month, it was not effective. She underwent standard surgery including a hysterectomy. She was thereafter free of disease 1 year after surgery. No estrogen receptor staining of the surgical specimen was observed, and 30% of the tumor was strongly positive for progesterone receptor. Direct DNA sequencing of exons 7 and 8 of *PTEN* and the K-*ras* codon 12 demonstrated the presence of no mutation. In addition, no dominant-negative p53 mutation was found by a yeast functional assay.

Conclusion: A uterine malignancy should therefore be included in the differential diagnosis in a young female patient complaining of abnormal genital bleeding.

Key Words: Endometrial carcinoma, Teenager, Conservative treatment, Gene mutation

(*Int J Gynecol Cancer* 2009;19: 896–897)

Endometrial carcinoma is extremely rare in teenagers. The youngest patient reported was 14 years,[1] and there is no report of a gene mutation associated with endometrial carcinomas in teenagers.

CASE REPORT

The patient was a 14-year-old Japanese female. Her menarche was at the age of 10, and her menstrual cycle was irregular. Her body mass index was 30 kg/m^2. She had never taken hormones of any kind, and she had never been pregnant. She had no complications such as hypertension and diabetes. She presented with menorrhagia and took a combination of estrogen and progestin for a month. However, the genital bleeding continued. Transrectal ultrasonography demonstrated a 35 mm-thick endometrium within an enlarged uterus and bilateral polycystic ovaries. An endometrial biopsy revealed grade 2 (architectural and cytologic grade 2) endometrioid adenocarcinoma. Computed tomography and magnetic resonance imaging showed no evidence of myometrial invasion or metastasis. The tumor measured 70 mm in diameter. Her serum cancer antigen 125 level was 11 U/mL. The serum luteinizing hormone, follicle-stimulating hormone, estradiol, and progesterone levels were 15.3 IU/L, 6.2 IU/L, 73.p mol/L and 1.9n mol/L, respectively, during the proliferative phase. An immunohistochemical examination showed that staining for the estrogen receptor (ER) and progesterone receptor (PgR) of the biopsy specimen was strongly positive in 60% and 90% (immunoreactive score were 9 and 12, respectively).[2] To determine whether the tumor was sensitive to progestin, she took 400 mg medroxyprogesterone acetate (MPA) a day for a month, and an endometrial curettage was performed. However, there was no change to suggest the effectiveness of MPA, such as the conversion of decidual reaction of stromal cells, subnuclear vacuolization, secretory differentiation, and regression of glandular cells (Fig. 1). Therefore, a hysterectomy, bilateral salpingo-oophorectomy, pelvic, and para-aortic lymphadenectomy were performed.

The pathological examination revealed a grade 2 endometrioid adenocarcinoma with squamous differentiation. A shallow

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Conflict of interest statement: The authors declare that they have no conflicts of interest.

ISSN: 1048-891X
DOI: 10.1111/IGC.0b013e3181a8342a

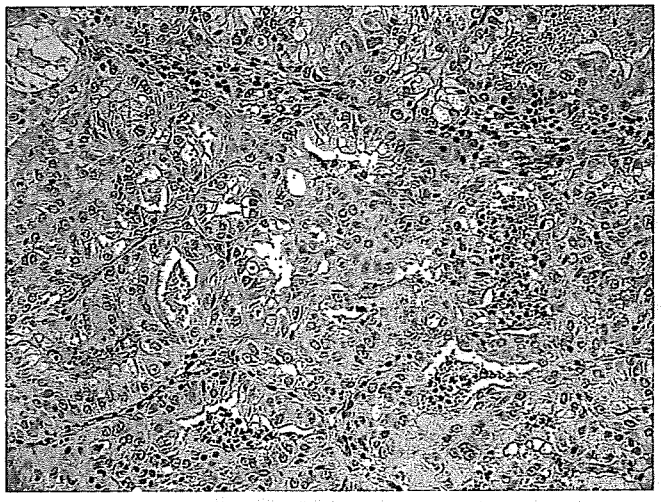

FIGURE 1. Endometrial curettage after hormonal therapy. Hematoxylin-eosin stain original magnification ×400.

myometrial invasion (less than one third) was seen. There was no cervical invasion or extrauterine disease. The fibrotic and thickened cortex of the ovaries and the atretic follicles suggested polycystic ovary syndrome. A small endometriotic focus was seen on the serosa of the uterus. The estrogen receptor was negative, and the PgR was strongly positive in 30% (immunoreactive score were 0 and 6) of the surgical specimen. Immunohistochemical examination revealed the following results: p53, epidermal growth factor receptor and human epidermal growth factor receptor 2 were positive in 90%, 90%, and 60%, respectively. No exon 7 or 8 mutation of *PTEN* and no codon12 mutation of K-*ras* were found by polymerase chain reaction and direct DNA sequencing. In addition, no dominant-negative p53 mutation was found by a yeast functional assay.[3] She had no recurrent disease for one year after the initial treatment without adjuvant therapy.

DISCUSSION

Endometrial carcinoma is frequently unsuspected in teenagers. Therefore, a diagnostic delay often occurs.[4] In the current case, the initial treatment started 2 months after her first visit.

The clinical features of this patient were similar to that of older women. She had polycystic ovary syndrome and obesity, both of which are associated with prolonged estrogen exposure, and she had no family history of hereditary nonpolyposis colorectal cancer. In addition, no mutation was found in any major carcinogenic or cancer suppressor genes. Therefore, hormonal factors may have contributed to the development of her disease.

Conservative treatment is recommended in patients with grade 1, International Federation of Gynecology and Obstetrics stage IA disease.[5–7] The patient and her family wished to preserve her fertility. In consideration of her fertility and age, conservative therapy was selected, although she had grade 2 disease. There is only 1 report of conservative treatment of teenage girls younger than 15 years. Farhi et al[8] reported 2 cases of 15-year-old teenage girls with grade 1, stage IA adenocarcinoma who underwent progestogen treatment and had no recurrent disease for 3 months and 10 years, respectively. In general, no response after 3 months of progestins is considered to indicate treatment failure.[9] However, no time-dependent histologic criteria of hormonal therapy for an endometrial carcinoma have yet been established. The existence of invasive cancer is suspected when the tumor is insensitive to progestin treatment even for 1 month.

In general, estrogen increases the target tissue responsiveness to itself and to progestins and androgens by increasing the concentration of its own receptor and that of the intracellular PgR. Progesterone, on the other hand, limits the tissue response to estrogen by blocking this mechanism, thus decreasing the concentration of ER.[10] In this case, the expression of both ER and PgR decreased after the administration of MPA. The tumor cells seemed to maintain the characteristics of a normal endometrium.

In conclusion, a uterine malignancy should therefore be included in the differential diagnosis in a young teenage girl complaining of abnormal genital bleeding, even if she is younger than 15 years.

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Survival and Failure Pattern of Patients With Endometrial Cancer After Extensive Surgery Including Systematic Pelvic and Para-Aortic Lymphadenectomy Followed by Adjuvant Chemotherapy

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Abstract: We investigated the survival and the failure pattern of 288 patients with endometrial cancer treated with extensive surgery including systematic pelvic and para-aortic lymphadenectomy followed by cisplatin-based chemotherapy from 1982 to 2002. We correlated the failure pattern with various clinicopathologic factors to find the predictors of recurrence sites. The 5-year overall survival rates were 97.5% for stage I, 87.5% for stage II, 85.2% for stage III, and 12.5% for stage IV. Notably, the 5-year survival rate was 76.5% for patients with stage IIIC disease. Among patients with a low risk (n = 92) for recurrence who received no adjuvant chemotherapy, 2 (2.2%) showed recurrent disease. Among those with intermediate (n = 98) and high (n = 98) risks for recurrence who received adjuvant chemotherapy, 9 (9.2%) and 20 (20.4%) showed recurrent disease, respectively. The recurrence sites were described as follows: distant (n = 12), vaginal (n = 8), peritoneal (n = 7), pelvic (n = 2), and lymphatic (n = 2). Lymphatic failure was found beyond the area of lymphadenectomy. Architectural and nuclear grades; myometrial, lymph-vascular space, and cervical invasions; and lymph node metastasis were predictors of distant failure. Cervical invasion and lymph node metastasis were predictors of vaginal failure. For patients with stage I/II cancer, the architectural and nuclear grades were related to distant failure. Seven (63.6%) of 11 patients with a low or intermediate risk survived after relapse, whereas only 1 (4.8%) of 21 patients with a high risk survived after a recurrence. We conclude that we need to further test the efficacy of systemic adjuvant therapy using new chemotherapeutic regimens to prevent distant failure and to improve the survival of patients with endometrial cancer.

Key Words: Endometrial cancer, Failure pattern, Chemotherapy, Pathologic risk factors

(*Int J Gynecol Cancer* 2009;19: 1585–1590)

The number of patients with endometrial cancer is increasing in Japan, the United States, and other countries.[1] Most patients with endometrial cancer are found out to have no clinical evidence of extrauterine spread (International Federation of Gynecology and Obstetrics [FIGO] stages I and II) and have a 5-year survival of approximately 90%. However, the number of patients with recurrent endometrial cancer is also increasing. Approximately, 10% to 15% of patients with early-stage endometrial cancer will experience recurrences.[2,3] To reduce the recurrence rate, adjuvant chemotherapy or radiotherapy has been applied. However, a definite standard therapy has not yet been established. For stage III to IV endometrial cancer, Randall et al[4] reported the results of a Gynecologic Oncology Group (GOG) randomized phase 3 trial of whole abdominal irradiation and platinum-doxorubicin (AP) chemotherapy. This study had a large impact on treatment because adjuvant therapy for advanced endometrial cancer had been limited mainly to radiotherapy, such as whole abdominal irradiation, pelvic irradiation, and vaginal brachytherapy.

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ISSN: 1048-891X
DOI: 10.1111/IGC.0b013e3181a833cc

TABLE 1. Clinicopathologic characteristics of 288 patients with endometrial cancer

Pathologic Variables	No.	%
FIGO stage		
I	164	56.9
II	24	8.3
III	92	31.9
IV	8	2.9
Histologic subtype		
Endometrioid	267	92.7
Nonendometrioid	21	7.3
AG		
G1	138	47.9
G2	109	37.8
G3	29	14.3
NG		
G1	130	45.1
G2	129	44.8
G3	29	10.1
MI		
None	36	12.5
≤1/2	146	50.7
>1/2	106	36.8
LVSI		
Negative/minimal	225	78.1
Moderate/prominent	63	21.9
CI		
Negative	227	78.8
Positive	61	21.2
Ovarian metastasis		
Negative	255	88.5
Positive	33	11.5
Pelvic node metastasis		
Negative	243	84.4
Positive	45	15.6
Para-aortic node metastasis		
Negative	264	91.7
Positive	24	8.3

AG, architectural grade, MI, myometrial invasion, NG, nuclear grade, CI, cervical invasion, LVSI, lymph-vascular space invasion.

Another important determinant for prognosis of patients with advanced endometrial cancer is the quality of surgery, especially the extent of lymphadenectomy. Metastatic involvement of retroperitoneal lymph nodes is one of the most important prognostic factors.[3] The association between the extent of lymph node involvement and survival has been demonstrated in most solid tumors. Chan et al[5] and Chan and Kapp[6] recently reported that the extent of lymph node resection improves the survival of patients with an intermediate/high risk of endometrioid corpus cancer.

We have routinely performed complete systematic pelvic and para-aortic lymphadenectomy in all operable patients with endometrial cancer because (1) nodal status is the most important prognosticator,[3] (2) results of lymphadenectomy allow tailoring of postoperative adjuvant treatment, (3) there is an apparent little survival advantage after lymphadenectomy,[6–8] and (4) there is no increased morbidity with lymphadenectomy.[6,7]

Although cytotoxic chemotherapy has been used primarily as palliative therapy for patients with advanced endometrial cancer, there is relatively little experience with postoperative systemic chemotherapy used as an adjuvant treatment in western countries. With the introduction of routine surgical staging, we have performed postsurgical cisplatin-based systemic chemotherapy in an adjuvant setting for patients with an intermediate or high risk of recurrence. Currently, only limited information is available about the survival benefit of the combination of extensive surgery and adjuvant chemotherapy, both of which might contribute to better outcome of advanced-stage endometrial cancer.

We, therefore, investigated the survival and failure pattern of patients with endometrial cancer to demonstrate the survival benefit of our treatment strategy consisting of extensive surgery including complete systematic pelvic and para-aortic lymphadenectomy and adjuvant chemotherapy in this retrospective analysis.

MATERIALS AND METHODS

A total of 303 patients with endometrial carcinoma underwent primary radical surgical treatment from 1982 to 2002 at the Department of Obstetrics and Gynecology, Hokkaido University Hospital. Two hundred eighty-eight patients, whose complete follow-up information was available, were registered in this retrospective study. All subjects underwent modified radical hysterectomy, bilateral salpingo-oophorectomy, and systematic retroperitoneal lymphadenectomy, consisting of complete dissection of pelvic and para-aortic lymph nodes from the femoral ring to the level of the renal vein. All lymphatic tissues that surrounded the arteries and veins were completely removed. Stage IV disease with distant metastasis (liver or lung metastasis) was excluded from this analysis. We, therefore, defined patients with stage IV endometrial carcinoma showing peritoneal metastasis. In this study, we defined the patients with a high or low risk for recurrence as those having stage III/IV disease or stage IA/IB with no/minimal lymph-vascular space invasion (LVSI), respectively. The others are classified as an intermediate-risk group. The median follow-up period was 74 months (range, 3–264 months). The patients' characteristics are shown in Table 1. The patients with an intermediate or high risk for recurrence were treated with adjuvant chemotherapy of 350-mg/m^2 cyclophosphamide, 40-mg/m^2 Adriamycin, and 50- to 70-mg/m^2 cisplatin (CAP) every 3 weeks.

The following histopathologic prognostic factors were included in this analysis: FIGO (1988) stage, histologic subtype, depth of myometrial invasion, architectural grade, nuclear grade, LVSI, ovarian metastasis, and lymph node metastasis (LNM). All risk factors were determined as previously described.[8]

Patterns of failure were recorded by the initial sites of failure (ie, vaginal, pelvic, peritoneal, lymphatic, and distant). In this study, when we divide the failure pattern into 2 groups, local failures included vaginal, pelvic, or lymphatic failure (pelvic or para-aortic), and distant failures included the recurrent disease in the lung, the liver, or the brain and lymphatic (other than pelvic or para-aortic) and peritoneal failures.

Statistics

Correlation between the variables was analyzed using a χ^2 test. Patient survival was calculated using the Kaplan-Meier method. The significance of the survival difference was examined using the log-rank test. $P < 0.05$ was considered statistically significant.

TABLE 2. Outcome of patients with recurrent disease according to the risk of recurrence

Risk	n	Cases of Recurrence (%)	Outcome	
			Alive (%)	Dead (%)
Low*	92	2 (2.2)	1 (50.0)	1 (50.0)
Intermediate†	98	9 (9.2)	6 (66.6)	3 (33.3)
High‡	98	21 (21.4)	11 (4.8)	20 (95.2)

*Stage IA/IB with no/minimal LVSI.
†Other than low or high risk.
‡Stage III/IV.

Statistical analyses were performed with the StatView software package (SAS Institute Inc, Cary, NC).

RESULTS

Survival Analysis and Initial Recurrence Sites After Extensive Surgery Followed by Adjuvant Chemotherapy in Patients With Endometrial Cancer

We analyzed the survival and relapse sites of 288 patients with endometrial cancer treated from 1982 to 2002 at Hokkaido University Hospital. Based on the results of postoperative pathologic examination, we gave adjuvant chemotherapy for patients with risks for recurrent disease, including 98 of an intermediate risk and 98 of a high risk. Ninety-two patients with a low risk for recurrence (stage IA or B with no or minimal LVSI) did not receive any adjuvant therapy.

Table 2 summarizes the recurrent disease of our patient cohort. A total of 32 patients (11.1%) experienced recurrent disease, including 2 (2.2%) of 92 patients with a low risk, 9 (9.2%) of 98 with an intermediate risk (stage IB, 2; stage IC, 5; and stage IIB, 2 patients), and 21 (20.4%) of 98 with a high risk (stage IIIA, 5; stage IIIC, 11, and stage IVB, 5 patients). Overall, 12 cases (37.5%) resulted in distant failure, 8 (25%) in vaginal stump failure, 7 (21.9%) in peritoneal failure, 3 (9.4%) in pelvic sidewall failure, and only 2 (6.3%) in lymphatic failure. Notably, lymphatic failure was found beyond the area of our lymphadenectomy (supraclavicular region). Among the patients with low or intermediate risks for recurrence, 11 patients (5.8%) revealed recurrent disease. No lymphatic failure was found in this group. Among the patients with a high risk for recurrence, 6 (28.6%) of 21 revealed distant failure, 6 (28.6%) vaginal stump failure, 5 peritoneal failure (23.8%), and 2 (9.5%) pelvic sidewall or lymphatic failure. Among 11 patients with stage IIIC endometrial cancer, we found 4 cases of distant failure (brain or liver), 3 of peritoneal failure, 3 of vaginal stump failure, and 1 of lymphatic failure (supraclavicular nodes). When we divided the failure patterns into 2 categories (distant or local), 8 (72.7%) of 11 patients revealed distant failure and only 3 (27.3%) of 11 showed local failure among the low- or intermediate-risk group, whereas 13 (61.9%) of 21 revealed distant failure and 8 (38.1%) of 21 showed local failure in the high-risk group. Seven (63.6%) of 11 patients with low or intermediate risk survived after relapse, whereas only 1 of 21 (4.8%) patients with a high risk survived after recurrence (Table 3).

During this treatment period, the 5-year overall survival rate was 97.5% for stage I, 87.5% for stage II, 85.2% for stage III, and 12.5% for stage IV. Notably, the patients with stage IIIC disease (node-positive patients) revealed a 5-year overall survival rate of 76.5% (Table 4A), apparently a better outcome compared with those in the FIGO annual report (Table 4B).[1]

TABLE 3. Initial site of recurrence

Initial Site of Recurrence	n (%)	Outcome	
		Alive (%)	Dead (%)
Distant	12 (37.5)	3 (25.0)	9 (75.0)
Vaginal stump	8 (25.0)	3 (37.5)	5 (62.5)
Peritoneum	7 (21.9)	2 (28.6)	5 (71.4)
Pelvic sidewall	3 (9.4)	0 (0.0)	3 (100.0)
Lymph node	2 (6.2)	0 (0.0)	2 (100.0)
Total	32 (100.0)	8 (25.0)	24 (75.0)

Pathologic Risk Factors for Failure Pattern

We correlated the various pathologic risk factors for recurrence, including histologic subtype, AG, NG, MI, CI, LVSI, ovarian metastasis, and LNM with the failure pattern to find the predictors (Table 5). We found that all factors analyzed except

TABLE 4. Five-year survival rates of patients with surgically staged endometrial cancer

Stage	5-Year Survival, %
A. Hokkaido University Hospital (1982–2002)	
I	96.9
IA	100.0
IB	97.8
IC	92.4
II	88.0
IIA	88.9
IIB	87.5
III	83.6
IIIA	92.8
IIIC	76.5
IV	11.5
IVB	11.5
B. Twenty-sixth FIGO annual report[1]	
I	89.6
IA	90.8
IB	91.1
IC	85.4
II	78.3
IIA	83.3
IIB	74.2
III	61.9
IIIA	66.2
IIIB	49.9
IIIC	57.3
IV	21.1
IVA	25.5
IVB	20.1

TABLE 5. Association of pathologic factors with distant or vaginal stump failure

Pathologic Variables	Distant Failure		P	Vaginal Stump Failure		P
	−	+		−	+	
Histologic type			0.21			0.11
Endometrioid	257	10		261	6	
Nonendometrioid	19	2		19	2	
AG			0.006			0.07
G1	137	1		137	1	
G2/3	139	11		143	7	
NG			<0.0001			0.19
G1/2	254	5		253	6	
G3	22	7		27	2	
MI			0.01			0.15
<1/2	179	3		179	3	
Ě1/2	97	9		101	5	
LVSI			0.02			0.07
Negative/minimal	219	6		221	4	
Moderate/prominent	57	6		59	4	
CI			0.01			0.01
Negative	221	6		224	3	
Positive	55	6		56	5	
Ovarian metastasis			0.15			>0.9999
Negative	246	9		248	7	
Positive	30	3		32	1	
LNM			0.03			0.03
Negative	233	7		236	4	
Positive	43	5		44	4	

histologic subtype and ovarian metastasis were significantly related to the distant failure.

Among the patients with stage I/II disease, AG ($P = 0.01$) and NG ($P = 0.004$) were significantly related to distant failure, and MI showed marginal significance ($P = 0.07$; Table 6). Cervical invasion and LNM were associated with the vaginal stump failure. No pathologic factors were correlated with the peritoneal failure (data not shown).

DISCUSSION

We examined the failure pattern in patients with endometrial cancer treated with extensive surgery including systematic lymphadenectomy followed by adjuvant chemotherapy (CAP) in this study. We found that distant failure is the most prevalent pattern of recurrence. We also determined the correlation between pathologic risk factors and the initial sites of recurrent disease in our patient cohort. We found that AG and NG are related to the risk for distant failure in patients with stage I/II disease. That only 3 cases revealed local failure and that no lymphatic failure was found in patients with stage I/II disease indicate 2 possibilities, including therapeutic efficacy of our surgical procedure, especially extensive lymphadenectomy and/or efficacy of adjuvant chemotherapy to reduce local recurrence for early disease. However, the number of recurrences is quite small in this study, which greatly limits the ability to identify accurate predictors of recurrence. We, therefore, further need to analyze larger number of cases to find more accurate predictors of recurrence in the near future.

Our systematic lymphadenectomy is extensive because we removed a median of 59 nodes in the pelvic region (range, 23–129) and a median of 24 nodes (range, 9–85) in the para-aortic area from 1991 to 2002 at our institute. Chan et al[5] and Chan and Kapp[6] reported that the extent of lymph node resection improves the survival of women with intermediate-/high-risk endometrioid uterine cancer; the subgroup receiving most extensive lymph node resection (>20) showed a significantly better 5-year disease-specific survival than other groups (1, 2–5, 6–10, and 11–20 nodes). Because we routinely removed more than 20 nodes, we do believe that our surgical procedure of lymphadenectomy might have some therapeutic effect for patients with endometrial cancer.

Radiotherapy and/or chemotherapy have been used as adjuvant therapies for endometrial cancer. Radiotherapy has been considered as a standard adjuvant therapy in western countries. The result of GOG 122, however, clearly demonstrated that adjuvant chemotherapy (Adriamycin and cisplatin) significantly improved progression-free and overall survivals than adjuvant radiotherapy (whole abdominal radiotherapy) for patients with stage III/IV disease,[4] indicating that chemotherapy should be considered as a standard adjuvant therapy for endometrial cancer. Systemic chemotherapy, as well as radiotherapy, has been widely accepted as a standard adjuvant therapy for endometrial cancer in Japan. A Japanese GOG (JGOG) study on the efficacy of whole pelvic radiotherapy versus cisplatin-based chemotherapy (CAP) revealed that CAP was significantly more effective for patients with a high/intermediate risk (stage II and IIIA, cytologically positive) than

TABLE 6. Pathologic risk factors related to distant failure in stage I/II endometrial cancer

Pathologic Variables	Distant Failure		*P*
	−	+	
Histologic type			0.23
Endometrioid	177	5	
Nonendometrioid	7	1	
Architectural grade			0.01
G1	101	0	
G2/3	83	6	
NG			0.004
G1/2	175	3	
G3	9	3	
MI			0.07
<1/2	131	2	
Ě1/2	53	4	
LVSI			0.58
Negative/minimal	160	5	
Moderate/prominent	24	1	
CI			0.58
Negative	160	5	
Positive	24	1	

whole pelvic irradiation.[9] In that report, the most prevalent sites of initial recurrence of distant failure are the lung and the liver in the CAP arm; this result was the same with that of our study. The results that vaginal relapse was more frequently seen in the CAP arm than in the whole pelvic irradiation (WPI) arm and that the rate of distant failure between the 2 arms was similar indicate that WPI is effective to reduce vaginal failure, chemotherapy (CAP) has similar adjuvant effect to WPI, or CAP is not very effective to reduce distant failure for intermediate-risk patents.

That distant failure is the most prevalent pattern of failure after adjuvant chemotherapy raises some questions. Is the dose of each drug used in our regimen appropriate for patients with endometrial cancer? Is CAP regimen the most effective to prevent distant failure? What is necessary to reduce distant failure leading to poor survival in the future? In the early 1990s, the CAP regimen was used as the standard chemotherapy for endometrial and ovarian cancers in Japan. Most Japanese gynecologists adopted CAP as the standard adjuvant chemotherapy rather than AP. Adriamycin has been used as a key drug for endometrial cancer. The dose of Adriamycin used for our patients was lower than in other trials using AP, such as the GOG study 107/122/177 (60 mg/m^2),[4,10,11] which might result in undesired distant failure. The result of this study also clearly indicates that we should consider a new chemotherapeutic regimen to further reduce distant failure leading to poor prognosis. Currently, the most promising chemotherapeutic drug for endometrial cancer is taxane. Indeed, the GOG 177 trial, which compared the efficacy of AP versus paclitaxel + Adriamycin + cisplatin (TAP) with granulocyte-colony stimulating factor support for patients with advanced or recurrent endometrial cancer, demonstrated that the TAP regimen significantly improved the survival of patients compared with AP,[11] clearly indicating that taxane is an active drug for advanced cases. In the clinical practice, however, paclitaxel + carboplatin has been widely used for endometrial cancer in Japan and in the United States. We need to wait for the result of the GOG 209 phase 3 trial to compare TAP with paclitaxel + carboplatin for advanced disease.

Table 4 reveals the overall survival of patients with surgically staged endometrial cancer treated in our institute and those reported in the FIGO annual report,[1] where adjuvant radiotherapy was selected roughly twice as often as adjuvant chemotherapy. Apparently, a better prognosis of the patients with stage III endometrial cancer treated in our institute indicates that our treatment strategy might be more effective to improve the survival of these patients. This is, at least in part, due to the extent of our surgical procedures described previously, which may result in a better treatment outcome of advanced cases and/or in adjuvant systemic chemotherapy instead of radiotherapy. However, we have to remember that survival data in FIGO were reported from diverse patient populations from centers in multiple countries and might not be an appropriate control group for comparison of survival analyses.

To achieve a better outcome of patients with endometrial cancer, we must improve the prognosis of advanced cases. For those patients, combined adjuvant chemotherapy with radiotherapy might be more beneficial.[12] We also need to investigate the efficacy of molecular targeting drugs alone or combination with other cytotoxic drugs to treat the patients with endometrial cancer. One of the possible agents is trastuzumab, a monoclonal antibody directed against HER-2, because *HER-2* gene has been reported to be overexpressed in some of uterine papillary serous carcinoma cases and related to poorer prognosis.[13] Further study on the efficacy of trastuzumab is necessary in clinical trial setting.

Among patients with stage I/II disease, AG and HG, which reflect the biological aggressiveness of malignant cells, were related to distant failure. This result indicates that molecular changes in aggressive tumor must be involved in the metastatic potential and/or probably in the resistance mechanism to adjuvant therapies. An attractive approach to identify the new predictors of metastasis and/or resistance to adjuvant therapies is a microarray-based analysis to compare the gene expression profile between nonmetastatic and metastatic or sensitive and resistant diseases in patients with similar clinicopathologic characters treated in the same manner.

In summary, combination of extensive surgery including systematic pelvic and para-aortic lymphadenectomy with systemic adjuvant chemotherapy might be beneficial to patients with advanced endometrial cancer, especially stage III disease. A new treatment strategy is also necessary to further improve the outcome of patients with advanced endometrial cancer.

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Local Biosynthesis of Estrogen in Human Endometrial Carcinoma through Tumor-Stromal Cell Interactions

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Abstract **Purpose:** The metabolism and synthesis of intratumoral estrogens are thought to play a very important role in the etiology and progression of endometrial carcinoma. Aromatase is a key enzyme in the conversion of androgens to estrogens, and aromatase localization studies have reported that aromatase immunoreactivity and mRNA were detected mainly in stromal cells. However, the effect of tumor-stromal interactions on local estrogen biosynthesis in endometrial carcinomas remains largely unknown.
Experimental Design: The endometrial carcinoma cell lines (Ishikawa and RL95-2) and breast carcinoma cell line (MCF-7) were cocultured with stromal cells isolated from endometrial carcinomas, and aromatization activity was measured using liquid chromatography-tandem mass spectrometry. We then confirmed the local biosynthesis of estrogens and tumor-stromal interactions on aromatase activity in Ishikawa and RL95-2 cells. In addition, we also examined the effects of aromatase inhibitors on cell proliferation.
Results: Aromatase activity was significantly higher in cocultures with Ishikawa or RL95-2 than in each monoculture, respectively. Estrone (E_1) concentrations were significantly higher than estradiol (E_2) concentrations in Ishikawa and RL95-2 cells, whereas E_2 was significantly higher than E_1 in MCF-7 cells. Cell proliferation was significantly inhibited in Ishikawa and RL95-2 cell cultures treated with aromatase inhibitors compared with control cultures.
Conclusions: These results indicate the contribution of not only E_2 but also E_1 to cancer cell proliferation in endometrial carcinoma. Our study may provide important information on metabolism and synthesis of intratumoral estrogens with regard to the etiology and progression of endometrial carcinoma, thus helping to achieve improved clinical responses in patients with endometrial carcinoma, who are treated with aromatase inhibitors. (Clin Cancer Res 2009;15(19):6028–34)

Endometrial carcinoma is one of the most common malignancies in developed countries, and its incidence has increased (1). *In situ* estrogen metabolism, including synthesis and degradation, has recently been thought to play a very important role in the development and progression of various human estrogen-dependent neoplasms. The results of several studies have shown that the concentration of estradiol (E_2) in endometrial carcinoma tissue was significantly higher than the concentration in normal endometrium (2). Our results from a previous study were generally consistent with those of other investigations; we found that E_2, estrone (E_1), and testosterone levels in tumor tissues were several times higher than concentrations measured in serum (3). These findings indicate that intratumoral estrogen metabolism and synthesis is important in the etiology and progression of endometrial carcinoma.

Intratumoral production of estrogen occurs as a result of the aromatization of androgens such as androstenedione and testosterone into estrogens, and it is catalyzed by the cytochrome *P*450 aromatase enzyme (4). An aromatase localization study has shown that aromatase immunoreactivity and mRNA were detected mainly in the stromal cells or fibroblasts of endometrial carcinoma but not in normal or hyperplastic endometrium (5). The reversible conversions of E_1 and E_2 are catalyzed by 17β-hydroxysteroid dehydrogenase (17β-HSD) types 1 and 2. The 17β-reduction of biologically less active E_1 is catalyzed to E_2 by 17β-HSD type 1 (6), and the oxidation of E_2 to E_1 is catalyzed by 17β-HSD type 2 (7). It was reported that 17β-HSD

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Received 4/22/09; revised 6/14/09; accepted 6/16/09; published OnlineFirst 9/29/09.
Grant support: Grant-in-Aid for Scientific Research on Priority Areas, Grant-in-Aid for Scientific Research, Grant-in-Aid for Young Scientists, and Grant-in-Aid for Exploratory Research from the Ministry of Education, Science, Sports and Culture, Japan; Grant-in-Aid from the Ministry of Health, Labor and Welfare, Japan; Global COE Program Special Research Grant (Tohoku University) from the Ministry of Education Science, Sports and Culture, Japan; Grant-in-Aid from the Kurokawa Cancer Research Foundation; Grant-in-Aid from All Japan Coffee Association; Japan Coffee Association; and Uehara Memorial Foundation.
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doi:10.1158/1078-0432.CCR-09-1013

Translational Relevance

Endometrial carcinoma is estrogen-dependent disease and standard therapy is established as staging laparotomy. However, progestin is only endocrine therapy for advanced endometrial carcinoma. Therefore, a more endocrine treatment is desirable. Estrogens are produced and accumulated by the conversion from androgens through aromatase pathway with tumor-stromal interactions. This is the first study to show the estrogen production using the coculture system. Aromatase activity in coculture was significantly higher than that in monoculture in endometrial carcinoma cells. Interestingly, estrone was significantly higher than estradiol in endometrial carcinoma cells, whereas estradiol was significantly higher than estrone in breast cancer cell line. In addition, we found significant inhibition for cell proliferation treated with aromatase inhibitors. Our study provided important information for the possible treatment in advanced or recurrent endometrial carcinoma patients. We might achieve better clinical responses in endometrial carcinoma patients with aromatase activity through decreasing local estrogen concentration by aromatase inhibitors (tailor-made medicine).

type 1 immunoreactivity and mRNA were absent in normal and hyperplastic endometrium and in endometrial carcinoma (8, 9), and 17β-HSD type 2 expression was detected in normal endometrium (secretory phase) but was decreased in hyperplastic endometrium and endometrial carcinoma (9). This is in contrast to the results of a study of these enzymes in breast cancer, in which nearly half of the cases showed 17β-HSD type 1 immunoreactivity in carcinoma cells, whereas 17β-HSD type 2 was not expressed (10). These reports have been shown that local estrogen biosynthesis was mainly regulated by aromatase and 17β-HSD types 1 and 2 in endometrial carcinoma (Fig. 1).

It is well known that aromatase expression is regulated by various transcriptional factors, including nuclear receptors and their putative ligands, in several types of human cells and tissues (11). However, the correlation between nuclear receptors and aromatase in parenchymal or carcinoma cells of endometrial carcinoma remains largely unknown. To evaluate the potential effects of tumor-stromal interactions on local estrogen biosynthesis in endometrial carcinoma, we established a coculture system using endometrial carcinoma cell lines and stromal cells (12). In the present study, we examine the local biosynthesis of estrogen and the effect of tumor-stromal interactions on aromatase expression and enzyme activity in endometrial carcinoma. In addition, we also investigate the effect of aromatase inhibitors on cell proliferation. In evaluating the metabolism and synthesis of intratumoral estrogens with regard to the etiology and progression of endometrial carcinoma, this study may help improve the way aromatase inhibitors are used to achieve better clinical responses in patients with endometrial carcinoma.

Materials and Methods

Patients and tissue preparations. A total of three endometrial carcinoma specimens were obtained from Japanese patients between 2004 and 2006 at the Department of Obstetrics and Gynecology, Tohoku University Hospital. This study was approved by the Ethical Committee of Tohoku University School of Medicine, and the required informed consents were obtained.

Cells and culture conditions. The human endometrial carcinoma cell line Ishikawa was kindly provided by Dr. Nishida (Tokyo Medical University Kasumigaura Hospital). The human endometrial carcinoma cell line RL95-2, the breast carcinoma cell line MCF-7, and the mouse fibroblast cell line 3T3-L1 were purchased from the American Type Culture Collection. Ishikawa, RL95-2, MCF-7, and 3T3-L1 cells were cultured in RPMI 1640 (Life Technologies) supplemented with 10% fetal bovine serum (BioWest), penicillin (100 units/mL), streptomycin (100 μg/mL), and amphotericin (250 ng/mL growth medium). Primary stromal cells employed in this study were designated #3, #11, and #16 and were isolated from human endometrial carcinoma tissue samples by collagenase treatment and maintained in RPMI 1640 with 10% fetal bovine serum, penicillin (100 units/mL), streptomycin (100 μg/mL), and amphotericin as described previously (13). mRNA expressions of aromatase and 17β-HSD type 2 in stromal cells were as follows: aromatase (+) and 17β-HSD type 2 (-) in #3 and aromatase (+) and 17β-HSD type 2 (+) in both #11 and #16.

Coculture system. For physical separation of stromal cells and the endometrial carcinoma cell lines, Transwell cultures were established in 6-well plates using Transwell permeable supports (0.4 μm pore; Corning). First, Ishikawa, RL95-2, MCF-7, 3T3-L1, and tumor stromal cells were cultured separately in 100-mm dishes until 70% confluence. Ishikawa, RL95-2, MCF-7, and 3T3-L1 cells were then cultured in Transwell chambers of the 6-well plates in the absence or presence of stromal cells, which were cultivated at the bottom of the 6-well plates. We named this system the coculture system as described previously (12). After 24 h of cultivation in the coculture system, the cell lines and stromal cells were separated again, and each component was examined in the aromatization assay, estrogen production assays, or by real-time quantitative reverse transcription-PCR (qRT-PCR). Cells types after monoculture or coculture were designated with the subscripts MO or CO, respectively ($Ishikawa_{CO}$, $\#3_{CO}$, $Ishikawa_{MO}$, etc.).

Before the assays, viable cells were counted by the trypan blue exclusion method, total RNA was extracted, and mRNA levels were determined by real-time qRT-PCR.

Total RNA extraction and cDNA synthesis. Total RNA was extracted from Ishikawa, RL95-2, MCF-7, 3T3-L1, and stromal cells using the RNeasy Mini Kit (Qiagen). A reverse transcription kit, SuperScript III Platinum CellsDirect Two-Step qRT-PCR kit with SYBR Green (Invitrogen), was used for the synthesis of cDNA.

Real-time qRT-PCR. Real-time qRT-PCR was carried out using the LightCycler TaqMan Master Ready-to-Use Hot Start reaction mix for PCR (Roche Diagnostics) for aromatase and the LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics) for the other mRNA targets using the LightCycler ST300 instrument (Roche Diagnostics). The primer sequences were as follows: forward 5′-CCTTCTGCGTCGTGTCATGCT-3′ and reverse 5′-GGAGAGCTTGCCATGCATCAA-3′ for aromatase, forward 5′-TGCTCAAGGAGGGAGTGC-3′ and reverse 5′-GGCGACAATTTCTGGTTCGGGTCAGGCATGGCATAG-3′ for estrogen receptor-α (ERα; ref. 14), forward 5′-ACCCAGCACAATGAAGAT-3′ and reverse 5′-GGTGTAACGCAACTAAGTCA-3′ for β-actin, forward 5′-TGCTGGACGTGAATGTAGTA-3′ and reverse 5′-GTATTGGAAGCGGTGGAAG-3′ for 17β-HSD type 1, and forward 5′-CAAAGGGAGGCTGGTGAAT-3′ and reverse 5′-TCACTGGTGCCTGCGATA-3′ for 17β-HSD type 2. The aromatase primers were designed using the Universal ProbeLibrary System (Roche Diagnostics). β-Actin primers were designed by the Nihon Gene Research Laboratories. 17β-HSD types 1 and 2 primers were

designed using OLIGO Primer Analysis Software (Takara Bio). The PCR conditions were 45 cycles (95°C for 10 s and 60°C for 35 s) for aromatase, 45 cycles (95°C for 10 s, 65°C for 10 s, and 72°C for 10 s) for ERα, 35 cycles (95°C for 10 s, 63°C for 10 s, and 72°C for 10 s) for β-actin, 45 cycles (95°C for 15 s, 60°C for 5 s, and 72°C for 10 s) for 17β-HSD type 1, and 45 cycles (95°C for 10 s and 70°C for 5 s) for 17β-HSD type 2. To quantify target cDNA transcripts, known concentrations of target gene cDNAs and the β-actin housekeeping gene were used to generate standard curves for real-time qRT-PCR. Each target mRNA level was expressed as the ratio to β-actin mRNA.

Aromatase assay. Aromatase assays were done on Ishikawa$_{MO}$, Ishikawa$_{CO}$, RL95-2$_{MO}$, RL95-2$_{CO}$, #16$_{MO}$, and #16$_{CO}$, with Ishikawa and RL95-2 cells using the 6a-methylandrost-4-ene-3,17-dione assay to quantify aromatization activity (15). The aromatase conversion of 6a-methylandrost-4-ene-3,17-dione, an androgen analogue, into an estrogen analogue (6a-methylestradiol) was shown to be highly specific, and an accurate evaluation of aromatase activity could be possible by measuring the production of estrogen analogue (15). 6a-Methylestradiol was measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) at ASKA Pharma Medical. The activity without cells was used as control.

Estrogen production assay. Ishikawa$_{CO}$, RL95-2$_{CO}$, and MCF-7$_{CO}$ were incubated at 37°C in fetal bovine serum–free RPMI 1640 containing 10 nmol/L androstenedione (Toronto Research Chemicals) or testosterone (Wako Pure Chemical Industries) as substrate for 24 h. Concentrations of E_1 and E_2 were evaluated by LC-MS/MS analysis at ASKA Pharma Medical as described previously (16). All the cells treated with substrate were quantified by the trypan blue exclusion method. The concentrations without cells were regarded as control.

Cell proliferation assay. After coculture with stromal cells for 24 h, Ishikawa and RL95-2 cells were trypsinized, harvested, and seeded in phenol red–free and fetal bovine serum–free medium with 10^{-8} mol/L testosterone in 96-well plates (5×10^{-4} cells/mL) for 24 h. We then added additional (10^{-8} mol/L) testosterone and aromatase inhibitors (10^{-6}-10^{-10} mol/L), which were either steroidal aromatase inhibitor, anastrozole (Toronto Research Chemicals), or nonsteroidal aromatase inhibitor, exemestane (LKT Laboratories). Ethanol was used as the vehicle. Cell proliferation was evaluated using the WST-8 method (Cell Counting Kit-8; Dojindo Molecular Technologies) at 0, 24, 48, and 72 h.

Statistical analysis. Data were analyzed using the *t* test for evaluation of two groups, employing the StatFlex 5.0 software program (Artec).

Results

Expression of aromatase, 17β-HSD types 1 and 2, and ERα mRNAs in Ishikawa, RL95-2, and MCF-7 cell cultures. The mRNA expression levels of 17β-HSD types 1 and 2, aromatase, and ERα were determined in Ishikawa, RL95-2, and MCF-7 (Fig. 2A-D) cell lines. For all cell lines, no significant differences were seen in mRNA expression levels for the enzymes and ERα between monoculture and coculture. 17β-HSD types 1 and 2 and aromatase expression were shown in all cell lines. ERα expression was also expressed in all cell lines.

Detection of aromatase activity in Ishikawa and RL95-2 cells. To estimate the interaction between cancer and stromal cells, aromatization activity in the coculture system was measured by LC-MS/MS. Our results showed that Ishikawa$_{MO}$, RL95-2$_{MO}$, and #16$_{MO}$ had aromatase activity. Aromatase activity was significantly higher in Ishikawa$_{CO}$, RL95-2$_{CO}$, and #16$_{CO}$ compared with Ishikawa$_{MO}$, RL95-2$_{MO}$, and #16$_{MO}$, respectively ($P < 0.05$, $P < 0.01$, and $P < 0.05$; Fig. 3).

Concentrations of estrogen with androgen as the substrate. Estrogen production in Ishikawa, RL95-2, and MCF-7 was measured using androstenedione or testosterone as the substrate by LC-MS/MS. Both E_1 and E_2 were produced in monoculture and coculture systems. E_1 levels of coculture were higher than those of monoculture in Ishikawa and RL95-2 and E_2 levels of coculture were higher than those of monoculture in MCF-7 (data not shown). In the case of androstenedione as the substrate, E_1 levels were significantly higher than E_2 in Ishikawa and RL95-2 ($P < 0.001$ and $P < 0.001$), whereas E_2 levels were significantly higher than E_1 in MCF-7 ($P < 0.001$; Fig. 4A).

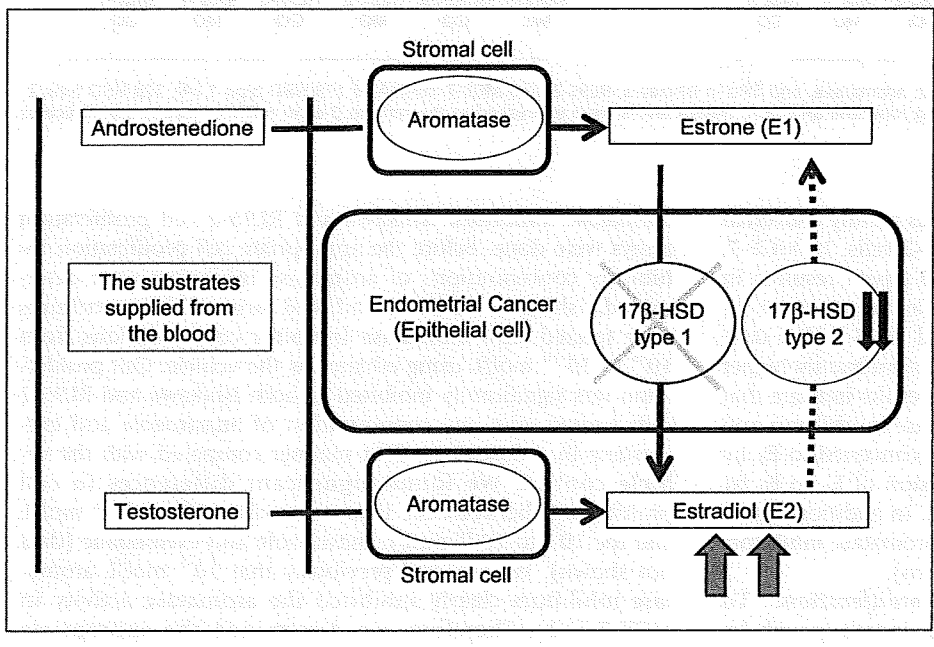

Fig. 1. Sketch of intratumoral estrogens metabolism and synthesis in endometrial carcinoma. Androgens as substrate are supplied from the blood. Androstenedione and testosterone are converted into E_1 and E_2 by aromatase mainly in stromal cells, respectively. 17β-HSD types 1 and 2 catalyze the reversible conversion of E_1 and E_2. The local regulation of estrogen activity is quite different between endometrial carcinoma and breast carcinoma. 17β-HSD type 1 was absent in endometrial carcinoma. 17β-HSD type 2 was expressed in secretary phase but decreased in endometrial carcinoma.

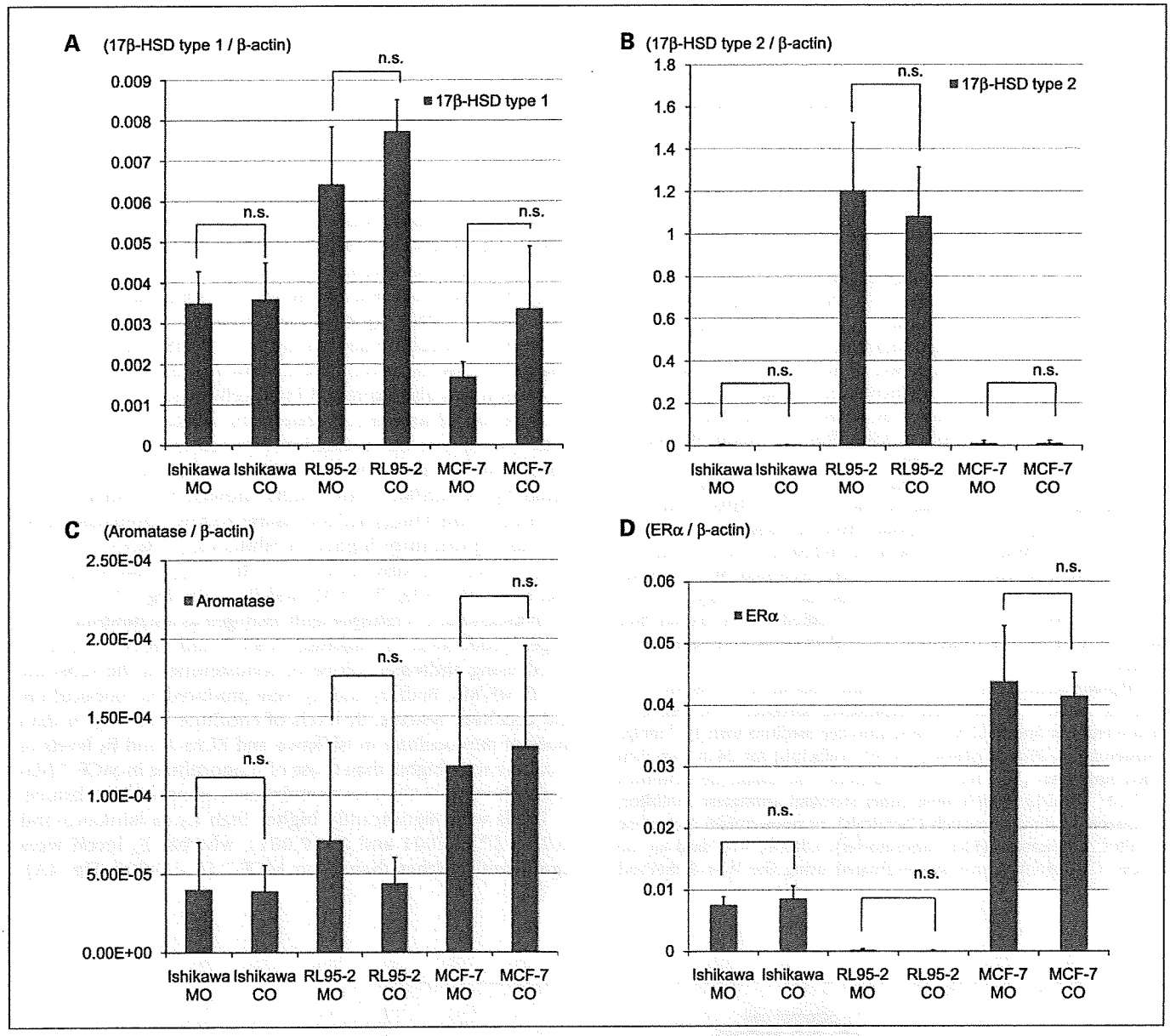

Fig. 2. mRNA expression of 17β-HSD types 1 and 2, aromatase, and ERα in Ishikawa, RL95-2, and MCF-7. mRNA of 17β-HSD type 1 (*A*), 17β-HSD type 2 (*B*), aromatase (*C*), and ERα (*D*) was measured using real-time qRT-PCR. mRNA levels were expressed as the ratio to β-actin mRNA. Results are triplicates of three independent experiments. Mean ± SE.

The results indicate that androstenedione was mainly converted to E_1 by aromatase in Ishikawa and RL95-2 cells. In MCF-7, higher conversion of E_1 to E_2 by 17β-HSD type 1 resulted in a high E_2 level. In the case of testosterone as the substrate, E_1 levels were higher than E_2 in Ishikawa and RL95-2 ($P < 0.01$ and nonsignificant), whereas E_2 levels were significantly higher than E_1 in MCF-7 ($P < 0.01$; Fig. 4B). The results indicate that testosterone was converted to E_2 by aromatase in Ishikawa and RL95-2 cells and stromal cells, and E_2 was converted to E_1 by 17β-HSD type 2. In MCF-7, lower conversion of E_2 to E_1 by 17β-HSD type 2 resulted in a high E_2 level. In addition, estrogen levels were markedly decreased by the aromatase inhibitors anastrozole and exemestane (data not shown).

Effect of aromatase inhibitors on cell proliferation. To estimate the inhibition of endometrial carcinoma growth by aromatase inhibitors, Ishikawa and RL95-2 cell proliferation assays were done. Before the appropriate cell proliferation inhibitory concentrations of aromatase inhibitors were determined, Ishikawa, RL95-2, and #16 stromal cell cocultures were treated with aromatase inhibitor concentrations from 10^{-6} to 10^{-10} mol/L using ethanol as the vehicle. Cell proliferation was significantly inhibited in both Ishikawa and RL95-2 cells treated with any concentration of anastrozole and exemestane in a dose-dependent manner compared with the vehicle control. We found significant differences in cell proliferation between the treatment with 10^{-6} to 10^{-8} mol/L and the 10^{-9} to 10^{-10} mol/L anastrozole and exemestane (data not shown). We reported previously that 10^{-8} mol/L aromatase inhibitors clearly inhibited the aromatase activity in MCF-7 (12). Therefore, we determined the appropriate

concentration of aromatase inhibitors as 10^{-8} mol/L, which was effective and less toxic. We employed six experimental coculture systems treated with 10^{-8} mol/L aromatase inhibitors, which included Ishikawa plus #3, #11, or #16 stromal cells and RL95-2 plus #3, #11, or #16 stromal cells. In addition, 3T3-L1 cells cocultured with stromal cells #3, #11, or #16 were used as controls because those cells do not express aromatase. We found significant differences in cell proliferation between any $Ishikawa_{CO}$ treated with 10^{-8} mol/L aromatase inhibitors and controls (Fig. 5A and B). We also found the same results between any $RL95\text{-}2_{CO}$ treated with 10^{-8} mol/L aromatase inhibitors and controls (Fig. 5C and D). There was no significant inhibition of cell proliferation in $3T3\_L1_{CO}$ treated with 10^{-8} mol/L anastrozole or exemestane.

To evaluate the effect of 17β-HSD type 2 on cell growth, cell proliferation rates were analyzed comparatively in the coculture system of Ishikawa and RL95-2 plus #3, #11, or #16 stromal cells, which had different expression patterns of 17β-HSD type 2. However, there were no significant differences seen in cell proliferation, although 17β-HSD type 2 expression varied (data not shown).

Discussion

The estrogens, especially E_2, which is a biologically potent estrogen, have been shown to contribute greatly to the growth and the development of estrogen-dependent tumors in endometrial carcinomas and breast carcinomas (17, 18). However, it is also true that the great majority of endometrial carcinomas occur during the postmenopausal period, when the ovaries no longer produce active sex steroids and circulating plasma estrogens are at very low concentrations. Numerous studies have reported that there was no consistent evidence of increased serum estrogen concentrations or other systemic estrogen abnormalities in women with endometrial carcinoma (19, 20). Tseng et al. and Yamaki et al. reported on the conversion of androgens to estrogens through aromatase in endometrial carcinoma (21, 22). In addition, Watanabe et al. have reported that aromatase protein and mRNA expression were predominantly detected in stromal cells (5).

In this study, we employed a coculture system that could provide important information regarding the intratumoral microenvironment, such as evaluation of cell-cell interactions (23). Aromatase activities in Ishikawa and RL95-2 cocultures were significantly higher than activities in each monoculture. The results indicate that aromatase activity was increased by tumor-stromal interactions. It has been reported previously that various aromatase-stimulating factors, such as interleukin-1, interleukin-6, interleukin-11, tumor necrosis factor-α, prostaglandin E_2, etc., are released from stromal or carcinoma cells in human breast carcinoma (24–26). However, in endometrial carcinoma, the possible effects of aromatase-stimulating factors

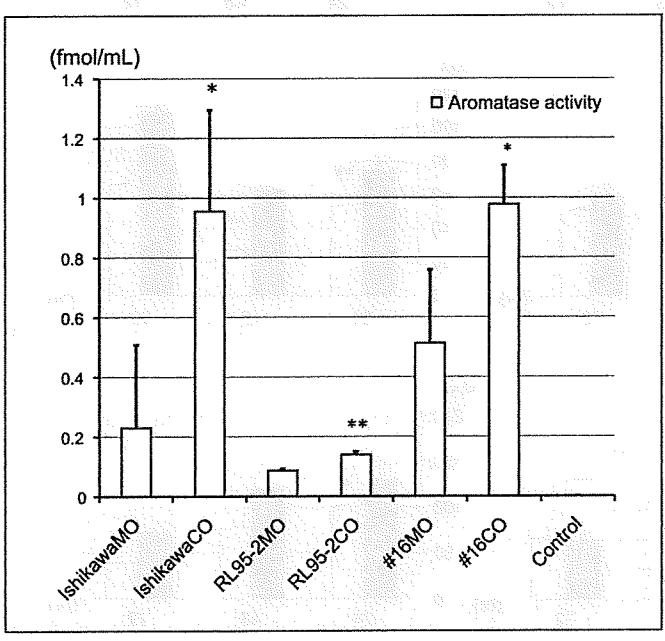

Fig. 3. Aromatase activity in $Ishikawa_{CO}$ and $RL95\text{-}2_{CO}$ with #16 stromal cells was significantly higher than that in $Ishikawa_{MO}$ and $RL95\text{-}2_{MO}$. Aromatase assay was done for $Ishikawa_{MO}$, $Ishikawa_{CO}$, $RL95\text{-}2_{MO}$, $RL95\text{-}2_{CO}$, $\#16_{MO}$, and $\#16_{CO}$ using 6a-methylandrost-4-ene-3,17-dione. The activity was measured by LC-MS/MS method. Mean ± SE. +, $P < 0.01$ versus $RL95\text{-}2_{MO}$; *, $P < 0.05$ versus $Ishikawa_{MO}$ and versus $\#16_{MO}$.

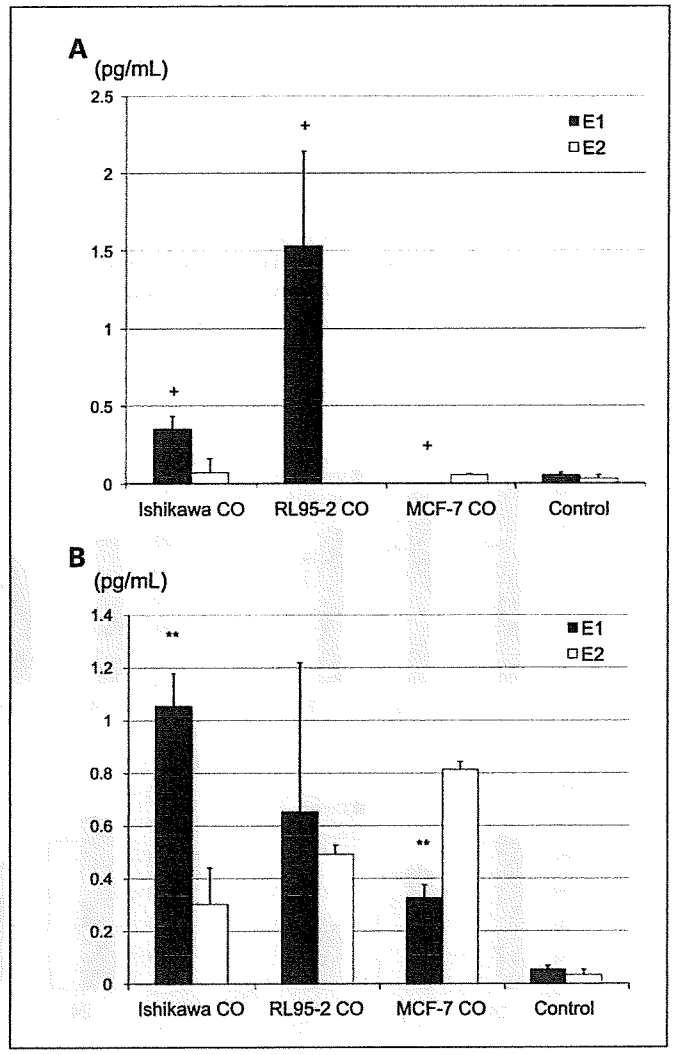

Fig. 4. Ratio of E_1/E_2, which was produced by aromatase, was contrary between Ishikawa, RL95-2, and MCF-7. Estrogen production assays were done treating with androstenedione (*A*) and testosterone (*B*) as substrate. We employed coculture system using Ishikawa, RL95-2, and MCF-7 with #16 stromal cells. Concentrations of E_1 and E_2 were measured by LC-MS/MS method. The buffer with androgen as substrate was regarded as control. Mean ± SE. **, $P < 0.01$ versus E_2; +, $P < 0.001$ versus E_2.

secreted from stromal cells on aromatase expression in stromal or carcinoma cells remain largely unknown. Further investigation is needed to elucidate their role in endometrial carcinoma.

It has been reported that intratumoral estrogen metabolism is different between endometrial carcinoma and breast carcinoma, although both of them are estrogen-dependent malignancies (27). 17β-HSD types 1 and 2 catalyze the reversible conversion of E_1 and E_2. 17β-HSD type 1 catalyzes the 17β-reduction of E_1 to E_2 (6), whereas 17β-HSD type 2 catalyzes the oxidation of E_2 to E_1 (7). It has been reported that 17β-HSD type 1 regulates the tissue concentrations of E_2 in breast carcinoma (28), and we have shown previously that 17β-HSD type 2 mainly regulates the tissue concentrations of E_2 and modulates estrogenic actions in normal endometrium and endometrial carcinoma (9).

This is the first study to measure aromatase activity and the amounts of estrogen production using coculture system and LC-MS/MS method in endometrial carcinoma cells. Previous studies have reported that androgen was converted into estrogen through aromatase in normal human endometrium and endometrial carcinoma tissues (21, 22). However, the aromatase activity and the amounts of estrogen production were

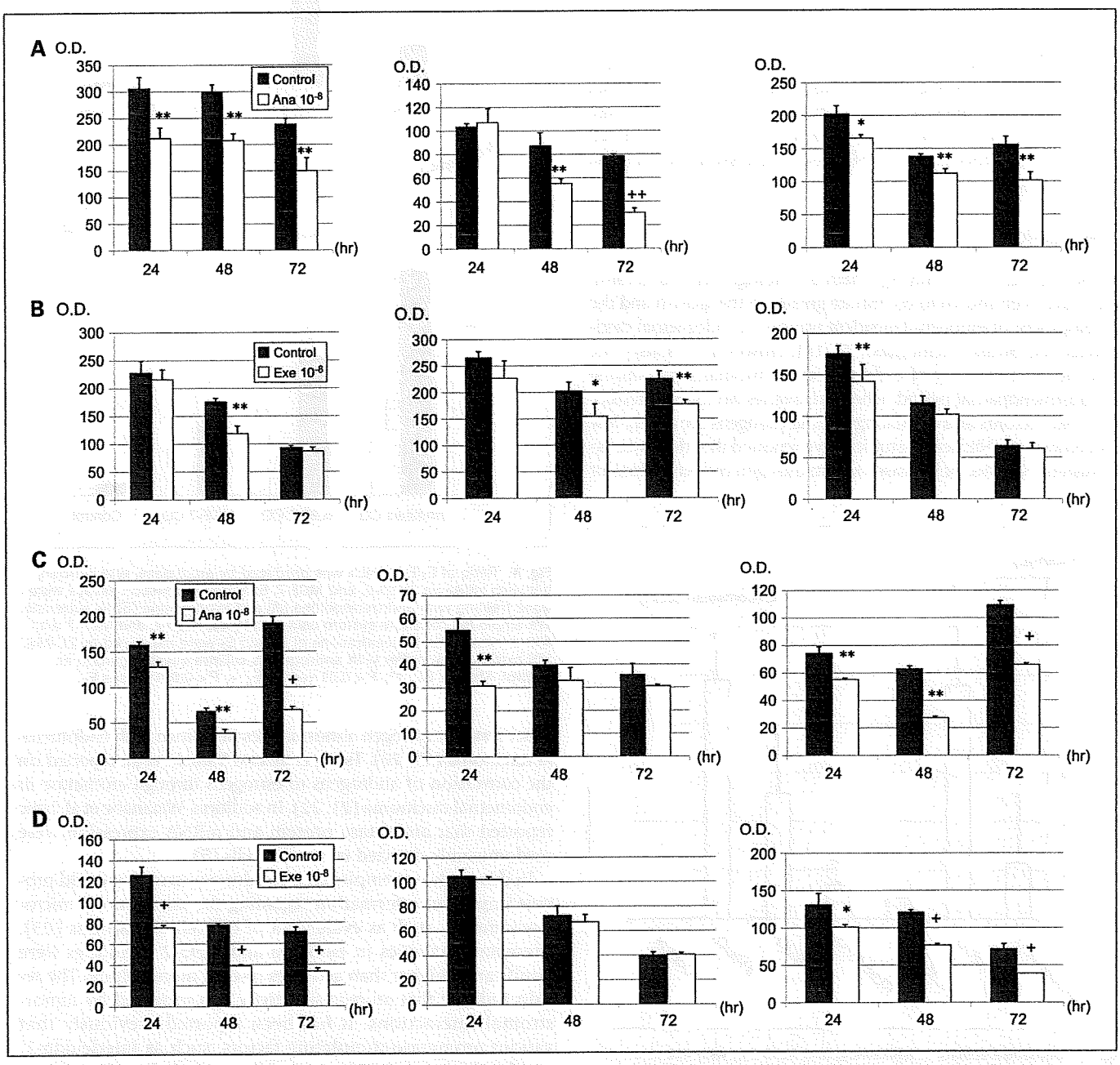

Fig. 5. Cell proliferation was significantly inhibited by aromatase inhibitors regardless of 17β-HSD type 2 expression. Cell proliferations of Ishikawa$_{CO}$ and RL95-2$_{CO}$ following treatment with 10^{-8} mol/L anastrozole (*Ana*) and exemestane (*Exe*) aromatase inhibitors. Cell proliferation was evaluated using WST-8 method. Mean ± SE. ++, $P < 0.0001$ versus control; +, $P < 0.001$ versus control; * *, $P < 0.01$ versus control; *, $P < 0.05$ versus control; *, $P < 0.05$ versus control. *A*, Ishikawa$_{CO}$ cell proliferation with anastrozole. *B*, Ishikawa$_{CO}$ cell proliferation with exemestane. *C*, RL95-2$_{CO}$ cell proliferation with anastrozole. *D*, RL95-2$_{CO}$ cell proliferation with exemestane.

not measured in detail. Our results to measure those values confirmed their reports on androgen conversion to estrogen through aromatase. In addition, we also measured the concentrations of E_1 and E_2 by LC-MS/MS. Interestingly, E_1 was significantly higher than E_2 in Ishikawa and RL95-2, whereas E_2 was significantly higher than E_1 in MCF-7. The results indicate that E_2 produced by aromatase was converted into E_1 by 17β-HSD type 2 and E_1 produced by aromatase was not converted into E_2 by low 17β-HSD type 1 in Ishikawa and RL95-2 cells but not in MCF-7 cells.

This study confirmed the importance of aromatase to estrogen metabolism and synthesis in endometrial carcinoma. The therapeutic use of aromatase inhibitors has been well defined for breast cancer. However, the therapeutic value of aromatase inhibitors to endometrial carcinoma is not clear. Some reports on the use of aromatase inhibitors for endometrial carcinoma have shown small or minimal effects, and these results were not dramatic and remain controversial (29, 30). However, those patients treated with aromatase inhibitor have not been checked for aromatase expression. To examine the possible effects of aromatase inhibitors on endometrial carcinoma, we first determined the expressions of aromatase and 17β-HSD type 2 in tumor stromal cells derived from several patients. Then we examined the effects of aromatase inhibitors on cell proliferation in Ishikawa and RL95-2 cocultures, in which the expression of aromatase and 17β-HSD type 2 varied. We found significant inhibition of cell proliferation using anastrozole and exemestane whether 17β-HSD type 2 was expressed or not. E_2 is biologically regarded as potent estrogen and we have reported that 17β-HSD type 2 might play some protective and/or suppressive roles against unopposed estrogenic effects by decreasing local estrogen activity (9). However, our results indicate the roles of not only E_2 but also E_1 in cancer proliferation.

In conclusion, we showed estrogen production through the aromatase pathway using androgen as substrate in the coculture system. Our study may provide important information on metabolism and synthesis of intratumoral estrogens with regard to the etiology and progression of endometrial carcinoma, thus helping to achieve improved clinical responses in patients with endometrial carcinoma, who are treated with aromatase inhibitors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Curr. Res. in Cancer 3, 2009 (13-27)

Heparanase expression and angiogenesis in endometrial cancer: Analyses of RT-PCR and immunohistochemistry

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Abstract

Background: The human heparanase has been shown to function in tumor progression, metastatic spread, and tumor angiogenesis. The aim of the present study was to assess heparanase expression in endometrial cancer in correlation with neovascularization and clinicopathological factors.

Materials and Methods: Fifty-two endometrial cancers were obtained from previously untreated patients (median age, 56 years, range, 35-80 years). The expression of heparanase mRNA was evaluated using a semi-quantitative reverse transcriptase-polymerase chain reaction and immunohistochemical staining (IHC) with anti-heparanase polyclonal antibody. This antibody was raised by immunizing a rabbit

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