Aromatase Expression in Stromal Cells of Endometrioid Endometrial Cancer Correlates with Poor Survival

Tomoya Segawa, Makio Shozu, Kouich Murakami, Tadayuki Kasai, Kazunori Shinohara, Kazuhiro Nomura, Satoshi Ohno, and Masaki Inoue
Department of Obstetrics and Gynecology, Kanazawa University School of Medicine, Kanazawa, Japan

ABSTRACT

Purpose and Experimental Design: To assess the prognostic significance of intratumoral aromatase in endometrioid endometrial cancer, sections from 55 patients with endometrial cancer were evaluated for expression of aromatase using immunohistochemistry, and the correlation between aromatase expression and clinicopathologic parameters were analyzed.

Results: Immunohistochemical staining for aromatase was positive for 32 (58%), 20 (36%), and 19 (34%) patients in cancer epithelial cells, stromal cells, and myometrial cells around the flank invasion, respectively. In situ hybridization also detected aromatase mRNA in all three types of cells. RT-PCR analysis revealed that aromatase mRNA was 2.5 ± 1.0 amol/μg total RNA (mean ± SE; n = 7) in tumor tissue. Western blot analysis detected the expected aromatase protein size of 58 kDa in cancer tissues more abundantly than in cancer-free endometrium (n = 3). The immunoreactivity in stromal cells correlated positively with advanced surgical stage and poor survival. Survival analysis revealed that the immunoreactivity of stromal cells was a significant prognostic factor, independent of histologic grade, muscular invasion, and lymph node metastasis, but dependent on surgical stage. By contrast, the immunoreactivity of aromatase both in cancer epithelial cells and myometrial cells did not correlate with prognosis.

Conclusions: To the best of our knowledge, this is the first evidence associating intratumoral aromatase expression in stromal cells and poor survival in endometrioid endometrial cancer. This positive linkage indicates that local expression of aromatase plays a role in tumor progression through the formation of in situ estrogens. In situ expression of aromatase may offer a potential target for management of endometrial cancers.

INTRODUCTION

Endometrioid endometrial cancer is the most frequent type of malignant neoplasm arising from the endometrium. They are usually estrogen receptor–positive and growth is promoted by unopposed estrogen. Several lines of clinical and epidemiological studies indicate that unopposed estrogen also contributes to the tumorigenesis of endometrioid endometrial cancer, in addition to the promotion of tumor growth (1). Although there is no consistent evidence of increased concentrations of serum estrogen in women with endometrioid endometrial cancer (2–4), the local concentration of estradiol in endometrial cancer tissues has been reported to be higher than that in blood (5) and in the endometrium of cancer-free women (6, 7). It is, therefore, conceivable that endometrioid endometrial cancer itself synthesizes estradiol in situ, which then contributes to self-growth and even tumorigenesis.

A conversion study showed that androstenedione is successfully converted to estrogen in endometrial cancer tissue at a higher rate than in normal endometrium (8). Expression of aromatase, a key enzyme for estrogen synthesis, has been detected in endometrial cancer using immunohistochemistry and RT-PCR techniques, whereas aromatase expression is low or not detected in endometrial hyperplasia (a putative precursor lesion of endometrioid endometrial cancer), as well as normal endometrium obtained from disease-free women (9, 10). These observations indicate that an increase in intratumoral aromatase plays a role in tumor progression, and consequently, poor prognosis. Only one study has analyzed the correlation between intratumoral aromatase expression and pathologic parameters of endometrial cancer, and in which analysis on outcome (prognosis and survival time) was not included (10).

The purpose of the present study was to assess the prognostic relevance of intratumoral aromatase in endometrioid endometrial cancer. We also examined whether cancer cells of glandular epithelium express aromatase in endometrioid endometrial cancer, as the expression of aromatase in cancer epithelial cells is a controversial topic in breast cancer, another well-known example of aromatase-expressing estrogen-dependent tumor (11–13).

MATERIALS AND METHODS

Patients and Samples. Tissues samples were obtained from 55 patients with endometrioid endometrial cancer who underwent surgical resection at Kanazawa University Hospital and Osaka University Hospital from 1990 to 2002 and who were not pretreated with neoadjuvant chemotherapy, hormonal therapy, or irradiation prior to tumor excision. This study was approved by the Institutional Review Boards and informed consent was obtained before the study. The tissue samples were fixed in 10% formalin, embedded in paraffin, serially cut into
4- to 6-μm-thick sections, and stained by routine histopathologic techniques. Histologic definition and grading were according to the WHO typing system. Surgical stage was determined in accordance with the International Federation of Gynecology and Obstetrics staging system. Patients with stage I disease \( (n = 40) \) were treated by total abdominal hysterectomy, bilateral salpingo-oophorectomy, and pelvic lymphadenectomy node dissection. Patients with stage II disease \( (n = 7) \) were treated by radical hysterectomy, bilateral salpingo-oophorectomy, and pelvic lymphadenectomy node dissection. In those stage I and stage II patients with pelvic lymph nodes positive for metastases and muscular invasion (more than 1/2 depth) and/or extensive vascular invasion, the above treatment was followed by additional irradiation of the whole pelvis with 50 Gy. Patients with stage III disease \( (n = 8) \) were treated by total abdominal hysterectomy and bilateral salpingo-oophorectomy followed by irradiation and combination chemotherapy. Patients who were treated according to a different regime were excluded from the study. The mean and SE of the follow-up period was 38 ± 29 months (range 2-130 months).

**Immunohistochemical Staining.** Immunostaining for aromatase protein was done using the CSA system (DAKO, Glostrup, Denmark) in formalin-fixed, paraffin-embedded tissue samples. Sections were dewaxed in xylene, and taken through a graded series of ethanol. After incubating with 0.3% hydrogen peroxide and protein block solution (DAKO) for 5 minutes, sections were treated with polyclonal antiaromatase antibody provided by Dr. Harada (Fujita Health University, Nagoya, Japan) for 15 minutes at room temperature after dilution to 1:1,000 in buffer. Negative controls included sections incubated with normal rabbit serum instead of the primary antibody. After incubation with the primary antibody, streptavidin-biotin complex and streptavidin-peroxidase complex were applied for 15 minutes each at room temperature. Sites of peroxidase activity were visualized with 0.02% hydrogen peroxide in PBS. Finally, the sections were counterstained with Nuclear Fast Red for 10 minutes, and the supernatant then centrifuged again for 60 minutes at 105,000 × g, with the resultant pellets washed once and then resuspended in buffer containing 50 mmol Tris-HCl, 20% glycerol, 1 mmol EDTA, and 1 mmol DTT. Microsom fractured were stored at −80°C until use. Microsomal protein (50 μg/lane) was electrophoresed and electroblotted onto a polyvinylidene difluoride membrane (Hybond P, Amersham, St. Louis, CA). Detection of aromatase signals was conducted as described using Enhanced Chemiluminescence Plus (Amersham, Aylesbury, United Kingdom; ref. 14).

**Reverse Transcription-PCR.** Total RNA was extracted using the Ultraspec RNA extraction kit (Biotech Laboratories, Houston, TX) according to the manufacturer’s instructions. The level of aromatase and glyceraldehyde-3-phosphate dehydrogenase mRNA was quantified by competitive quantitative RT-PCR using a synthetic RNA as an internal standard as described (15). The primers used for PCR amplification was as follows: AROM203 \( (5'-GCCGAATGAGCTGTAAT-3') \) and AROM205 \( (5'-CTCCTCAGGGCTTTTC-3') \) for aromatase; and GAPDH21F \( (5'-ACGTCGAGGCACAT-"TTT-3') \) and GAPDH21R \( (5'-GCAAGCTTCCCGTTCAG-3') \) for glyceraldehyde-3-phosphate dehydrogenase. The level of expression was calculated based on the ratio of the target intensity to that of the internal standard as described (15). The quality of total RNA was guaranteed by a similar quantitative analysis of glyceraldehyde-3-phosphate dehydrogenase mRNA. The fidelity of the PCR product was confirmed by sequencing using an automatic sequencer (ABI Prism 310 genetic analyzer, PE Applied Biosystems, Foster City, CA).

**Statistical Analysis.** Statistical analyses were done using the unpaired Student’s \( t \) test for comparison of means between the two groups. Fisher’s test was employed for the comparison of percentages between the groups. The cumulative probabilities of survival and their confidence interval at 95% (95% CI) were estimated using the Kaplan-Meier method, after which we carried out a multiple regression analysis using Cox’s proportional hazard model. Statistical analyses were carried out using the StatView 5.0 software program (SAS Institute, Inc., Cary, NC).
RESULTS

Detection of Aromatase Expression. Of the 55 patients, immunohistochemical staining for aromatase was positive for 32 (58%), 20 (36%), and 19 (34%) patients in cancer cells, stromal cells, and myometrial cells, respectively. Although expression of immunoreactive aromatase in the three cell types correlated with each other in individual patients, quite a few cases showed a discrepancy in staining between cell types: for example, more than half (17 cases) of the cases in which the cancer cells were positive for aromatase (32 cases) did not express aromatase in stromal cells. Similarly, 17 of 32 cases in which the cancer cells were positive for aromatase did not express aromatase in myometrial cells, and 8 of 20 cases in which stromal cells were positive for aromatase did not express aromatase in myometrial cells.

Western blotting analysis was done on three patients for whom surrounding normal-appearing endometrium was simultaneously available for the analysis. The expected 52-kDa aromatase was detected in all three patients, and the amount of aromatase protein was greater in cancer tissue than in normal-appearing endometrium (Fig. 1). Aromatase mRNA in frozen cancer tissue samples was 2.5 ± 1.0 amol/μg total RNA (mean ± SE; n = 7; range, 0.05-6 amol/μg total RNA), which corresponded roughly to the level of aromatase expressed in leiomyoma tissues, another example of aromatase-expressing neoplasm of the uterus (14).

To validate aromatase expression in cancer cells, in situ hybridization analysis was conducted on tissues samples obtained from three patients whose tissue was fixed with paraformaldehyde solution immediately after resection and for no more than 24 hours. A positive aromatase mRNA signal was observed in cancer cells as well as stromal cells in two of the three patients analyzed, which corresponded to the localization identified by immunohistochemistry (Fig. 2). Weak staining was also detected by in situ hybridization in myometrial cells in all three cases examined.

Relationship between Aromatase Expression and Clinicopathologic Features. The correlation between aromatase expression and clinicopathologic parameters was analyzed using the χ² test. Parameters included in the analysis were as follows: age (≤60 or >60 years), menarche (≤12 or >12 years), menopause (premenopause or postmenopause), gravidity (0 or ≥1 pregnancy), parity (0 or ≥1 labor), obesity (body mass index; ≤24 or >24), diabetes mellitus (absent or present), hypertension (absent or present), personal history of cancers (absent or present), and familial history of cancers (absent or present). Positive association with aromatase staining was found for only two parameters: obesity and a personal cancer history. Positive staining for aromatase expression in cancer cells was inversely correlated with obesity (odds ratio, 0.27; 95% CI, 0.09-0.85; P = 0.03) and a personal cancer history including double cancers (odds ratio, 0.15; 95% CI, 0.03-0.82; P = 0.03). Positive staining for aromatase expression in stromal cells was associated with a history of cancer in the family, although with marginal significance (odds ratio, 3.38; 95% CI, 1.04-10.98; P = 0.07). There was no clinical parameter associated with aromatase expression in myometrial cells.

Among the pathologic parameters, positive aromatase-staining in stromal cells correlated with poor survival and advanced surgical stage (Table 1). Aromatase staining in cancer epithelial cells and myometrial cells did not show any correlation with the pathologic parameters analyzed.

Survival Analysis. As shown in Fig. 3, a univariate survival analysis revealed that patients with aromatase-positive stromal cells had poor survival compared with the patients with aromatase-negative stromal cells (P = 0.04, log rank test). Survival did not discriminate between positive and negative aromatase staining for cancer cells and myometrial cells. Other clinical parameters, including advanced surgical stage (stage I versus stage II or III), myometrial invasion (≤1/2 versus 1/2), lymph node metastasis (negative versus positive) and advanced histologic grade (G3 versus G1 or G2), which are known prognostic factors of endometrioid endometrial cancer, significantly affected the survival rates in our patients, assuring the validity of the patient group enrolled in our study (P in log rank test was 0.001, 0.01, 0.04, and 0.001, respectively).

Prognostic factors were also analyzed by the multivariate Cox proportional-hazard model. Among four variables, histologic grade G3 (versus G1 or G2) and aromatase-positive stromal cells (versus negative staining) were identified as independent prognostic factors (Table 2). However, aromatase expression of stromal cells was no longer a significant prognostic factor when the International Federation of Gynecology and Obstetrics surgical stage was included in the analysis.

DISCUSSION

In our series of 55 patients with endometrioid endometrial cancer, stromal immunoreactivity of aromatase in tumor tissue correlated positively with advanced surgical stage and poor prognosis. Survival analysis showed that stromal immunoreactivity was a significant prognostic factor, independent of histologic grade, muscular invasion, and lymph node metastasis, but dependent on surgical status.

Considering the growth-promoting role of estrogen, it is conceivable that aromatase-expressing stromal cells produce estrogen in situ, which acts on cancer epithelial cells through a paracrine route, thereby supporting their proliferation. In fact, estradiol concentrations in endometrial tumor tissues are reportedly higher than that in cancer-free endometrium and correlate positively with the surgical stage of disease (7), and thus, presumably, are related to prognosis. The role of intratumoral estrogen on tumor progression has been well recognized in breast cancer: in situ estrogen produced by stromal cells of breast cancer tissue and the surrounding adipose

![Fig 1 Western blot analysis of aromatase in endometrioid endometrial cancer tissues. Three sets of microsome fractions were prepared from paired tissue samples of cancer tissue (Ca) and normal-appearing endometrium (EM). Microsome fractions (50 μg/lane) were electrophoresed and blotted onto the membrane. A microsome fraction (0.1 μg/ lane) prepared from placenta (Pl.) was used as the positive control.](image-url)
tissue support tumor cell growth and consequently influence prognosis (16). It is believed that aromatase expression of stromal cells is maintained by local factors secreted by breast cancer epithelial cells, such as some cytokines and prostaglandin E2 (17). It would be interesting to examine whether a similar interrelationship between stromal cells and cancer epithelial cells exists in endometrial cancer.

The positive correlation that we found between aromatase expression and poor prognosis does not necessarily indicate a causative relationship between the two. The increase in aromatase expression may simply be a tissue reaction to the severe stromal invasion, and neither the cause of tumor progression nor the cause of poor prognosis. This might be supported by the findings that the strong immunoreactivity of aromatase is often found in close vicinity to flank invasion (10). Thus, we cannot exclude the possibility of false linkage between intratumoral aromatase expression and poor prognosis at this point.

We detected both immunoreactivity and mRNA of aromatase in all three cell types (cancer, stromal, and myometrial cells) in the cancer-bearing uterus. Watanabe et al. (10) localized aromatase expression in endometrial cancer tissues to stromal cells and myometrial cells, but not to cancer epithelial cells, using antibodies supplied by the same source and a hybridization.
Thus, the different localization to aromatase-expressing cell types between Watanabe et al.’s study and our study might be attributable to a difference in antibody batches and lots, the fixation method of tissue samples, technique of staining, and skill in histologic analysis. Watanabe et al. (10) considered aromatase staining in cancer epithelium as a nonspecific cross-reaction of antibodies, primarily based on their in situ hybridization results. By contrast, our in situ hybridization detected an aromatase mRNA signal in some cancer epithelial cells, as well as in stromal cells. A similar controversy has been noted for localization of aromatase in breast cancer (13, 18, 19), and completion of an international collaboration to develop aromatase antibodies is warranted (20).

If cancer epithelial cells, as well as stromal cells, express aromatase, it is unclear as to why staining of cancer cells did not correlate with prognosis, whereas stromal staining did. There are several possible explanations. As the number of stromal cells is usually greater than that of epithelial cells and greater numbers of precursors are available for stromal cells in the vicinity of blood vessels than for epithelial cells, the quantity of estrogen produced by stromal cells would be greater than that produced by epithelial cells. Another possibility is that the aromatase signal detected in epithelial cells was that of nonfunctional forms of aromatase, as we did identify several splicing variants of aromatase that had truncated functional domains, such as the heme domain on exon 10 (data not shown). Possible contributions of these variants are currently under investigation.

In the present study, obesity was negatively correlated with local expression of aromatase in cancer cells. A similar trend was detected for aromatase in stromal cells, although the correlation failed to reach statistical significance (odds ratio, 0.33; 95% CI, 0.10-1.12; \( P = 0.09 \)). One hypothesis explaining this negative correlation is that the increased level of circulating estrogen reported in obese women compensates for the decreased local aromatase expression in cancer tissue or vice versa. Both the level of circulating estrogen and that of local aromatase expression will have to be determined in endometrial cancer patients as well as cancer-free controls to validate this hypothesis.

The precursor for peripheral aromatization is most likely androstenedione in women, as it is the most abundant androgen in circulation in premenopausal and postmenopausal women (9). As the direct product of the aromatizing reaction of androstenedione is estrone, cooperation of 17\( \beta \)-hydroxysteroid dehydrogenases is essential for conversion into fully biologically active estrogen, i.e. estradiol. In fact, interconversion activity has been reported in endometrial tissue (21). Expression of type 2 17\( \beta \)-hydroxysteroid dehydrogenases has been reported in glandular epithelial cells of cancer-free and malignant endometrium, whereas expression of type 1 17\( \beta \)-hydroxysteroid dehydrogenases has not been detected (22, 23). The expression of other types of 17\( \beta \)-hydroxysteroid dehydrogenases has not

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**Table 2** Multivariate analysis of prognostic factors for survival based on the Cox proportional hazard regression model

<table>
<thead>
<tr>
<th>Variable</th>
<th>Four covariables</th>
<th>Two covariables</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RR</td>
<td>95% CI</td>
</tr>
<tr>
<td>Histologic grade G3 (vs. G1 or G3)</td>
<td>2.74</td>
<td>2.17-34.24</td>
</tr>
<tr>
<td>Aromatase positive in stroma (vs. negative)</td>
<td>4.01</td>
<td>1.18-13.69</td>
</tr>
<tr>
<td>Node metastasis (vs. no metastasis)</td>
<td>2.96</td>
<td>0.80-10.99</td>
</tr>
<tr>
<td>Muscular invasion ( \geq 1/2 ) (vs. &lt;1/2)</td>
<td>2.74</td>
<td>0.99-9.39</td>
</tr>
<tr>
<td>FIGO stage II-III (vs. stage I)</td>
<td>4.35</td>
<td>1.36-13.96</td>
</tr>
</tbody>
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Abbreviations: FIGO, International Federation of Gynecology and Obstetrics; CI, confidence interval.
been examined. The precise identification of type(s) of 17β-hydroxysteroid dehydrogenases function in normal and cancer tissues of the endometrium remain to be determined. Testosterone, a reduced form of androgen at the 17β position secreted from the ovary and therefore a direct precursor of estradiol, might serve as an alternate to androstenedione in the ovaries of postmenopausal women (24, 25). The testosterone concentration in ovarian veins is higher in women with endometrial cancer than in women free from endometrial cancer (26). Because of the unique anatomic proximity of the ovary and the uterus, and anastomozing ovarian and uterine vessels, testosterone secreted from the postmenopausal ovary may constitute a significant portion of the precursors of estradiol, which contribute to tumorigenesis of endometrial cancer (27). It has been noted that androgen secretion is increased from ovaries showing stromal hyperplasia (24, 28, 29), and women with endometrioid endometrial cancer show ovarian stromal hyperplasia more frequently than women without endometrial cancer (28, 30).

Aromatase inhibitors would be potential agents for endocrine therapy and prevention of endometrial cancer (31). In vitro treatment of endometrial cancer tissues with aromatase inhibitors showed that depletion of in situ estrogen results in decreased cell proliferation of tumor cells (32). In vivo use of aromatase inhibitors dose-dependently inhibits aromatase activity in human endometrial cancers (33). Safety data from a clinical trial for postmenopausal women with breast cancer testing anastrozole and tamoxifen, alone or in combination, also indicated that the use of anastrozole, an aromatase inhibitor, reduced the incidence of endometrial cancer: a calculation of standard incidence rates for patients taking anastrozole was 0.73 relative to an age-matched and regionally compared standard population (34). A recent study by Rose et al. (35) was, however, unable to show distinct clinical efficacy with an aromatase inhibitor (anastrozole): only 2 of 23 patients showed a partial response to aromatase inhibitor therapy. The number of patients included in that study was small and only 9 of the 23 patients had endometrioid endometrial cancer, an estrogen-related type of malignancy where depletion of in situ estrogen might theoretically be of therapeutic benefit (27). The results of linkage between in situ expression of aromatase and prognosis indicates that the possible use of aromatase inhibitors in endometrioid endometrial cancers remains to be ascertained in a large number of cases.

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REFERENCES

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