A Large-Scale Gene Expression Comparison of Microdissected, Small-Sized Endometrial Cancers with or without Hyperplasia Matched to Same-Patient Normal Tissue

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ABSTRACT

Purpose: Type I endometrial cancer is accompanied by hyperplasia and type II endometrial cancer is not. The purpose of our study is to identify genes involved in carcinogenesis of endometrial cancer, especially those differentially expressed by type I and type II cancers.

Experimental Design: Using a cDNA array technique, we examined expression of 1176 cancer-related genes in endometrial cancer cells sampled from 21 tumors with a diameter of <10 mm, and we compared the expression patterns of the tumor cells with expression patterns of corresponding normal endometrial cells. Of these, 10 cases were type I cancers, and 11 cases were type II cancers. Laser capture microdissection directed precise separation of cells of interest from stromal cells. In cancer cells relative to normal cells, we identified genes that were commonly up- and down-regulated. Then we identified genes differentially expressed by the two types of cancer. Finally, in situ protein expression of some of these gene products was examined using immunohistochemistry.

Results: Of 1176 genes examined, 32 genes were upregulated, and 58 were down-regulated in cancer cells (P < 0.05). Between the two types of cancer, 45 genes were highly expressed in type I cancers, and 24 were highly expressed in type II. Immunohistochemistry confirmed that P-cadherin expression was cancer specific, and vascular endothelial growth factor-C and MLH1 expression were limited to type I and type II cancers, respectively.

Conclusions: A more accurate way of assessing gene expression during endometrial carcinogenesis shows evidence of providing candidate genes for use in conquering endometrial carcinoma.

INTRODUCTION

Endometrial cancer is a common malignancy of the female genital tract, of which two subtypes are described previously (1, 2). Hyperplastic endometrial regions (those lacking cellular atypia) surround type I endometrial cancers, which are usually well differentiated and respond favorably to appropriate therapy. In contrast, type II endometrial cancers are not associated with hyperplastic endometrial regions, are often poorly differentiated, and are more likely to metastasize and yield a poor prognosis.

As with other cancers, endometrial cancer is thought to develop as a result of a multistep process of oncogene activation and tumor suppressor gene inactivation, and there have been a number of reports describing alterations of these genes in various endometrial cancers (3–6). Analysis using cDNA expression array techniques has made the simultaneous analysis of thousands of genes from in vitro and in vivo specimens feasible (7–12). Even more accurate expression analysis can result when cancer cell populations are homogeneously separated from stromal cells and analyzed using LCM.6 Thus, for identification of genes involved in and in common between the two pathways of endometrial carcinogenesis, the combined means of cDNA expression array with LCM, allows a promising, systematic approach to expression analysis of thousands of genes. Caveats for developing an understanding of endometrial carcinogenesis include: consideration that type I cancers arise from hyperplastic endometrial regions, whereas type II cancers do not; and taking into account that gene expression analysis from cells in small-sized, early-stage tumors is preferable to that in advanced cancer cells because expression profiles will likely change during tumor progression.

In the present study, we examined expression of 1176 cancer-related genes in 21 endometrial cancers (10 type I cancers and 11 type II cancers). We sampled cancer tissues from tumors with a diameter < 10 mm, as well as from normal endometrium of the same individual, and analyzed these using cDNA expression arrays after LCM. Initially, we compared the

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The abbreviations used are: LCM, laser capture microdissection; VEGF-C, vascular endothelial growth factor-C; MMP, matrix metalloproteinase; STAT, signal transducers and activators of transcription.
gene expression profiles of cancerous cells with those of normal endometrial cells from the same individual and identified genes likely to be commonly involved in both types of endometrial cancer. Next, we explored genes differentially expressed in the two types of cancer.

**MATERIALS AND METHODS**

**Tissue Preparation and LCM.** Fresh specimens of endometrial cancer tissues and normal endometrium were obtained with the informed consent of 21 patients who underwent hysterectomy. These specimens were frozen in liquid nitrogen and stored at −80°C. All specimens were derived from small-sized tumors with a diameter < 10 mm. Cancer tissues were cut into sections with a thickness of 10 μm and mounted on uncoated glass slides. One of these sections was stained with H&E by the standard method and was examined by a gynecologic pathologist to confirm the histological types. To determine the tumor

<table>
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<tr>
<th>Table 1</th>
<th>Patients profiles of the small endometrial carcinomas</th>
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<tbody>
<tr>
<td></td>
<td>Type I hyperplasia (+)</td>
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<tr>
<td>Age (mean)</td>
<td>43–55 (49 yrs)</td>
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<tr>
<td>Surgical stage</td>
<td></td>
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<tr>
<td>Ia</td>
<td>4</td>
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<td>Ib</td>
<td>2</td>
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<td>Ic</td>
<td>1</td>
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<td>IIa</td>
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<td>IIc</td>
<td>0</td>
</tr>
<tr>
<td>Histologic diagnosis</td>
<td></td>
</tr>
<tr>
<td>Endometrioid adenocarcinoma G1</td>
<td>9</td>
</tr>
<tr>
<td>Endometrioid adenocarcinoma G2</td>
<td>1</td>
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<tr>
<td>Endometrioid adenocarcinoma G3</td>
<td>0</td>
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<tr>
<td>Serous adenocarcinoma</td>
<td>0</td>
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<td>Prognosis</td>
<td>0/10 (0%)</td>
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![Separation of the endometrial cancer cells and adjacent normal endometrial cells in type I and II endometrial cancer by LCM. Sections were stained with H&E. A, type I (with hyperplasia) endometrial cancer. The cancer lesion (T) is surrounded by the hyperplastic endometrium (H) with adjacent normal endometrium (N). B, high power view of the type I cancer lesion with hyperplastic lesion. Diagnosed as endometrioid adenocarcinoma grade I. C, the type II (without hyperplasia) endometrial cancer. The cancer lesion is with adjacent normal endometrium. D, high power view of the type II cancer lesion without hyperplastic lesion. Diagnosed as endometrioid adenocarcinoma grade II. E, and M, frozen section of the normal endometrial tissue from A before LCM (E) and after LCM (M) and normal endometrial cells captured on LCM cap (M). F, J, and N, frozen section of the type I cancer lesion from B before LCM (F) and after LCM (J) and type I cancer cells captured on LCM cap (N). G, K, and O, frozen section of the normal endometrial tissue from C before LCM (G) and after LCM (K) and normal endometrial cells captured on LCM cap (O). H, L, and P, frozen section of the type II cancer lesion from D before LCM (H) and after LCM (L) and type II cancer cells captured on LCM cap (P). Scale bar = 100 μm.](https://cancerceres.aacrjournals.org)
size, entire cut sections were made from all of the operation materials. We studied only tumors that were <10 mm in diameter. Histopathological classification of each tumor was performed according to WHO classification (13). We have separated these small endometrial carcinomas into two subtypes: type I is carcinoma with hyperplasia and type II is carcinoma without hyperplasia (13). Patient profiles were obtained from medical records. Clinical stage was determined according to the International Federation of Gynecology and Obstetrics stage (14).

Before LCM, sections were fixed in 70% ethanol for 1 min at a room temperature, followed by stepwise dehydration with 95% ethanol twice and 100% ethanol twice for 1 min, and incubation in xylene for 1 min at a room temperature. After the section was air-dried, cancer cells were selectively microdissected by the PixCell II LCM system (Arcturus Engineering, Mountain View, CA) following the manufacturer’s protocols (15, 16). In all patients, corresponding normal endometrial cells were also dissected from normal endometrium to allow analysis of the differential expression between cancer cells and normal endometrial cells.

**RNA Extraction and T7-Based RNA Amplification.** Laser-captured cell nests were resuspended with RLT lysis buffer (Qiagen, Valencia, CA), and total RNA was extracted using the RNeasy Kit (Qiagen) according to the manufacturer’s protocol. During this step, DNase I (Roche, Basel, Switzerland) was added to remove any contaminating genomic DNA. Total RNA was subjected to T7-based amplification, as described previously (17). Two rounds of T7-based amplification were performed.

**Expression Array Method.** Except for cDNA probe synthesis, hybridizations to cDNA expression array membranes were performed by using Atlas Human Cancer 1.2 Array system (BD Biosciences Clontech, Palo Alto, CA) basically according to the manufacturer’s protocol. Before cDNA probe synthesis, 5 μg of antisense RNA were reverse transcribed using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) so that the CDS primer provided with the system could anneal to the 3'-untranslated region of specific genes in the resultant single-stranded DNA and allow for extension of the cDNA probe by DNA polymerase I. To synthesize cDNA probe, the resultant DNA was purified by Min Elute PCR purification kit (Qiagen) and incubated with DNA polymerase I in the presence of 35μCi of [32P]-dATP and CDS primer mixture. Then the synthesized probes were purified with QIA quick PCR purification kit (Qiagen). Hybridization and washing was performed according to the manufacturer’s protocol as described previously (18, 19).

**Array Analysis.** Hybridized and washed membranes were exposed to a BAS imaging plate (Fujifilm, Tokyo, Japan) for 48 h, and the plate was scanned using Bioimaging analyzer BAS 2500 system (Fujifilm). The quantification of spot intensities, qualities, and subtraction of local background was performed by using Atlas Image Software (version 2.0; BD Biosciences Clontech). To compare signal intensities of each gene in different samples, each gene signal was normalized to the 90th percentile of all signals on each membrane by setting the 90th percentile to 1.

**Hierarchical Clustering.** The logarithmic values of 42 samples (half cancerous and half normal) were analyzed using Genespring 5.0 software (Silicon Genetics), which provided average-linkage hierarchical clustering. The distance metric used in this analysis was the Pearson’s correlation coefficient. To obtain reproducible clusters, we selected only the 111 genes that passed the cutoff filter (normalized signals were >0.6 in >34 samples).

**Selection of Genes with Different Expression.** We first compared expression levels of each gene between cancer cells and normal endometrial cells of the same patient. We identified genes as being commonly up-regulated in either cancer cells type according to the two-tailed paired Student’s t test (P < 0.05). When comparing gene expression between the two types of cancer, we calculated the expression ratio of the signal in cancer cells to the signal in normal endometrial cells (T/N); log-transformed ratios were used in the statistical analysis. The cutoff value was set at 0.3-fold of the 90th percentile of each membrane, and the gene signals <0.3 were replaced with 0.3. Because the data were unreliable for spots with intensities replaced with 0.3 for both signals in cancer cells and those in normal endometrial cells, genes corresponding to these spots were eliminated from the data set. We used only 474 genes that passed the cutoff filter (either signal in cancer cells or that in...
normal endometrial cells was $>0.3$ in $\leq 14$ of 21 total cases). Genes with different expression in cancer than in normal endometrial cells were identified using the one-tailed unpaired Student's $t$ test ($P < 0.05$).

**Immunohistochemistry.** Four-μm thick sections from the formalin-fixed and paraffin-embedded tissue sample were deparaffinized and dehydrated through a graded ethanol series (100% ethanol twice and 95% ethanol twice for 1 min each), incubated in 10 mM citrate buffer (pH 6), and heated in a microwave oven for 5 min at 500 W. After treatment with methanol containing 0.3% H$_2$O$_2$ to inhibit endogenous peroxidase, the sections were incubated with primary antibodies against P-cadherin (clone: 56; BD Transduction Laboratories, Lexington, KY) and MLH1 (clone: G168-15; BD Biosciences PharMingen, San Diego, CA) and polyclonal antibody against VEGF-C (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for 12 h. Antibody-antigen complexes were detected by the streptavidin-biotin technique (Nichirei SAB-PO kit, Tokyo, Japan). Color was developed with 0.03% 3,3′-diaminobenzidine tetrahydrochloride (Merck KGaA, Darmstadt, Germany) in 50 mM Tris-HCl buffer (pH 7.6) containing 0.006% H$_2$O$_2$. Countstaining was performed with hematoxylin. Negative controls omitted the primary antibody and substituted PBS.

**Evaluation of Immunohistochemical Results.** The percentage of positive cells was evaluated on 10 consecutive high magnification power fields ($\times 40$) by two observers. Mean values were obtained from 10 counts/tissue section. Protein expression was evaluated by grading the results as follows: strongly positive, when over 70% of the epithelial cells were stained; positive, when 30–70% of the epithelial cells were stained; heterogeneously positive, when up to 30% of the epithelial cells were stained; and negative, when 0% of the cells were stained.

**RESULTS**

**Profiles of 21 Endometrial Cancer Patients.** From 1997 to 2001, 367 patients underwent hysterectomies to remove endometrial cancer at the Cancer Institute Hospital in Tokyo. Patients with small tumors with a diameter of $<10$ mm made up 46 of these cases, and informed consent was obtained from 21 of these 46 cases. Patient profiles from those 21 cases are shown in Table 1. Of the 21 cases, 10 cases were type I cancer (with
hyperplasia), and 11 cases were type II cancer (without hyperplasia). Cases were considered sporadic because none of the patients met the Amsterdam criteria for hereditary nonpolyposis colorectal carcinoma syndrome (20). None of the patients had received preoperative hormone therapy, chemotherapy, or radiation therapy. No significant differences in age, surgical stage, body mass index, parity, gravidity, or serum levels of CA19-9 and CA12-5 were seen between the two types of cancer (Table 1, data not shown). In histological diagnosis, most patients (9 of 10) were diagnosed as endometrioid adenocarcinoma grade 1 in type I group, whereas only 1 of 11 patients was in the type II group. Four of the remaining 10 patients were diagnosed as endometrioid adenocarcinomas grade 2, 2 as grade 3, and 4 as serous adenocarcinomas in the type II group. Moreover, the type II group seemed to have poorer prognosis with 1 patient who died of endometrial cancer and 2 in whom the disease recurred, whereas neither death nor recurrence was shown for patients in the type I group.

Gene Expression Analysis of Endometrial Cancer Cells and Normal Endometrial Cells by Using cDNA Expression Arrays. To identify genes involved in molecular mechanisms of endometrial carcinogenesis, we used a cDNA ex-
pression array technique that allowed us to examine expression of 1176 cancer-related genes in cancerous and normal endometrial cells collected from the same patient. We looked at the two corresponding cell types collected from the 21 patients described above. To accomplish an accurate profiling of gene expression, cancer cells and normal endometrial cells were microdissected from fresh endometrial cancer tissues and adjacent normal endometrial tissues using LCM (Fig. 1). Total RNA was then extracted, amplified by T7 RNA polymerase, and subjected to cDNA expression array analysis (for details, see “Materials and Methods”). Hierarchical clustering was applied to these data without discriminating between cancer and normal cells. As shown in Fig. 2, both cancer and normal samples from the same patients tended to cluster together (e.g., nos. 11N and 11T, nos. 13N and 13T, nos. 15N and 15T, nos. 1N and 1T, nos. 18N and 18T, nos. 19N and 19T, and nos. 16N and 16T), indicating that overall profiles of gene expression in the cancer cells were similar to those in normal cells from the same patient.

**Genes with Differential Expression between Endometrial Cancer Cells and Normal Endometrial Cells.** To identify candidate genes that may be involved commonly in endometrial carcinogenesis, we first explored genes with differential expression levels in cancer and normal cells regardless of the cancer type. As shown in Fig. 3A, we used a two-tailed paired Student’s t test (P < 0.05) to select 32 genes that were up-regulated in cancer cells. Among them, the most significantly up-regulated gene in the cancer cells was c-myb. Notably, genes involved in cell cycle regulation such as cyclin-dependent kinase regulatory subunit 1 and 2, cyclin D2, CDC25A, 14-3-3s, and CDK inhibitor p19INK4d were also significantly up-regulated. P-cadherin was also up-regulated in cancer cells. Next, we selected 58 genes that are down-regulated in other cancers (P < 0.05; Fig. 3B). Among them, the most significantly down-regulated gene in the endometrial cancers was stem cell factor. Unexpectedly, MKI67, previously reported to be involved in cell proliferation, was down-regulated in the endometrial carcinomas (21). In addition, apoptosis signal-related genes such as tumor necrosis factor-related apoptosis-inducing ligand receptor and caspase-2, integrins (α E, β 8, and α 1), and ubiquitin showed down-regulation.

**Genes with Differential Expression in Type I and Type II Endometrial Cancers.** The molecular mechanism of carcinogenesis in type II cancer may differ from that in type I cancers because type I cancers are associated with hyperplastic endometrial regions, whereas type II cancers are not. To understand endometrial carcinogenesis more precisely, we went on to isolate genes with differential expression in the two types of cancer. For these analyses, the expression level of each gene in the cancer cells was normalized to that in the corresponding normal cells by calculating the T/N ratio (a ratio of tumor tissue to normal tissue gene expression from the endometrium of an individual). As shown in Fig. 4, 69 genes were found to have different levels of expression in type I and type II cancers. Of these, 45 genes exhibited higher expression levels in the type I group relative to the type II group; these included three oncogenes (MMP11, RHOG, and platelet-derived growth factor B subunit precursor), three transcription factors (STAT2, octamer-binding transcription factor 1, and GATA-6), growth factor VEGF-C precursor, caspase (caspase-1/IL-1 β converting enzyme), and so on. Conversely, the remaining 24 genes exhibited higher expression levels in the type II group than in type I group. Interestingly, these included several genes associated with DNA replication and repair such as the DNA primase small subunit, proliferating cell nuclear antigen, MLH1, O6-methylguanine-DNA methyltransferase, DNA polymerase α catalytic subunit, and Ku (p70/p80) subunit, as well as several transcription activators, including PIRIN, early growth response protein 1 (hEGR1), STAT1, IFN regulatory factor 1, and transforming protein p21/K-ras 2B (KRAS).

**Immunohistochemical Staining of P-Cadherin, VEGF-C, and MLH1.** The signal intensity on the cDNA expression arrays basically reflects the extent that an mRNA is expressed in the tested cells. Greater expression of an mRNA implies that its gene product is also strongly expressed. On the basis of our expression array analysis, we chose three of the genes described above that showed potential as possible markers for common, type I and type II cancers, to follow in cells at the protein expression level using immunohistochemical staining; these were, respectively, P-cadherin, VEGF-C, and MLH1. As shown in Fig. 5, P-cadherin up-regulation was common to both types of endometrial cancers, which agrees with the cDNA expression array data; it was strongly expressed in the cytoplasm of both type I and II endometrial cancers but not in their corresponding normal endometrium. The cDNA expression array showed that VEGF-C was up-regulated in type I endometrial cancers, and likewise, its protein was strongly expressed selectively in the cytoplasm of the type I cancer cells but only weakly expressed in the cytoplasm of noncancerous cells (Fig. 6). The DNA mismatch repair gene MLH1, which we considered up-regulated in type II cancers according to the cDNA expression array did show, at the protein level, positive selectively in the nucleus of the cancer cells in type II cancers, whereas the type I (with hyperplasia) endometrial cancer cells demonstrated either negative or low staining for MLH1 (Fig. 7). However, normal endometrium corresponding to either the type I and type II sample groups showed a fair amount of staining, too. This immunohistochemical data suggested that MLH1 expression, therefore, was down-regulated in type I group rather than being up-regulated in the type II group. These results showed that protein expression of P-cadherin, VEGF-C, and MLH1 determined immunohistochemically correlated with expression of their mRNAs as determined by cDNA expression arrays, and they point to the use of these proteins as markers for endometrial cancers.

**DISCUSSION**

In this study, we examined the expression of 1176 cancer-related genes in endometrial cancer cells and their corresponding normal endometrial cells from 21 patients by using cDNA expression arrays and LCM. We identified one set of genes differentially expressed in cancer cells and normal cells and another set of genes differentially expressed in type I cancers with hyperplasia and type II cancers without hyperplasia. Three of these genes were selected for immunohistochemical analysis. This additional analysis revealed that the expression of P-
cadherin, which was up-regulated in both cancer types as determined by cDNA array analysis, was specific to the cytoplasm of cancer cells. Similarly, the expressions of VEGF-C and MLH1 were limited to type I and type II cancers, respectively, which agreed with the results of cDNA expression arrays. We expect that these three genes, and many of the other discriminating genes identified here via cDNA arrays that await immunohistochemical confirmation, may eventually prove to be markers for endometrial cancers and will lead to delineation of common and divergent pathways in endometrial tumorigenesis.

Recently, Risinger et al. (12) examined the expression patterns of 35 endometrial cancers and 7 normal endometria by cDNA microarray and isolated genes expressed differently in endometrioid and nonendometrioid cancer and in cancer and normal cells. However, there are critical differences between our present study and theirs; first, we classified endometrial cancers as type I with hyperplasia and type II without hyperplasia, not as endometrioid and nonendometrioid cancers. Thus in our study, type I cancer samples are all endometrioid adenocarcinomas, but type II cancer samples included 7 endometrioid adenocarcinomas and 4 serous adenocarcinomas. Second, all of the samples we studied are derived from small-sized tumors with a diameter < 10 mm to gear the analysis of gene expression toward earlier stages of tumor progression. Third, we...
virtually purified the endometrial cancer cells and normal endometrial cells by LCM before analysis; as a technique, we have shown that the precision of LCM is key to effectively eliminating spurious expression by contaminating cells found in bulk cancer tissues (19). Fourth, we analyzed gene expression in both cancer cells and the corresponding normal endometrial cells from each patient. Hierarchical clustering analysis revealed that overall profiles of gene expression in cancer cells are similar to that in normal cells from the same patient (Fig. 2). Therefore, we normalized the expression level of each gene by calculating a T/N ratio before identifying genes that discriminate between type I and type II cancers. Clearly, these considerations have improved the accurate identification of those genes with differential expression levels in endometrial cancer cells.

In this study, we identified many genes that were up-regulated and down-regulated in endometrial cancer cells relative to normal, patient-matched endometrial cells. The most significantly up-regulated gene was c-myb. Previous studies demonstrated that c-myb is overexpressed in estrogen receptor-positive endometrial cancer and breast cancer cells (22, 23). Moreover, c-myb oncogene is amplified in hereditary BRCA1 breast cancers (24). P-Cadherin, which we found to be up-regulated in endometrial cancer cells as determined by cDNA expression arrays, also showed increased protein levels as determined immunohistochemically. P-Cadherin is a member of cadherin family proteins, which are integral membrane glycoproteins that mediate calcium-dependent cell-cell adhesion. Cell adhesion molecules are considered to play an important role in invasion and metastasis of the cancer. P-Cadherin expression was previously reported in gastric cancer, colon cancer, breast cancer, and cervical adenocarcinoma (25-28). Furthermore, in cervical adenocarcinoma, this protein is expressed preferentially in invasive as opposed to in situ lesions (28). Thus, P-cadherin could play a role in endometrial cancer development.

Several studies describe alteration of oncogenes and tumor suppressor genes that appear to be specific for type I or type II cancers. Type I cancers are characterized by mutation of PTEN, KRAS, and defects in DNA mismatch repair, and type II cancers often contain mutation of TP53 (29-31) and HER2/NEU (32). Microsatellite instability, caused by defects in DNA mismatch repair, is seen in 17–23% of endometrial cancers (6, 33, 34). Simpkins et al. (35) reported that methylation of MLH1 promoter and a lack of detectable MLH1 are important in endometrial tumorigenesis. In our present study, MLH1 expression was down-regulated selectively in type I cancer cells (Fig. 4), which agreed with the previous study that microsatellite instability was seen predominantly in type I cancers. In the present study, we observed an interesting down-regulation of other genes related to DNA damage signaling and DNA repair such as O6-methylguanine-DNA methyltransferase, DNA polymerase α catalytic subunit, and Ku (p70/p80) antigen in type I cancer cells that might prove to be affecting DNA repair in type I cancers. On the other hand, we saw that VEGF-C was up-regulated in type I cancer cells at both the mRNA and protein levels in comparison with type II cancers (Figs. 4 and 6). VEGF-C binds to its receptors FLT-4 (VEGF receptor-3) and KDR (VEGF receptor-2) and induces proliferations of endothelial cells of blood vessels and lymphatic endothelial cells, respectively (36). In
gastric cancer and endometrial cancer, VEGF-C is closely related to lymphatic invasion and lymph node metastasis (37–39), but absolute confirmation of VEGF-C involvement in type I endometrial cancer remains to be established.

In addition to the three genes described above, we detected expression of genes previously reported to be involved in endometrial cancer or other cancers. KRAS, which was significantly up-regulated in type II group in this study, is reported to be mutated at codon 12 in 26% of endometrioid adenocarcinomas but in only 2% of serous adenocarcinomas (40), and another group reported that 11% of the endometrial cancers had KRAS point mutations that were equally common in tumors with and
without hyperplasia (6). In addition, Enomoto et al. (41) reported that activation of \textit{KRAS} by point mutations plays a significant role in the pathogenesis of endometrial cancer. \textit{MMP11} was up-regulated in type I group cancers in this study. MMPs belong to a gene family of zinc-dependent endopeptidases and are considered to be involved in the degradation of the extracellular matrix substrate in association with tumor cell invasion. Enhanced production and activation of MMP7 was reported in type II endometrial cancers (42). In immunohistochemical studies, MMP11 expression was reported in ovarian

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**Fig. 7** Immunohistochemical staining of the tumor and the normal endometrium with anti-MLH1 antibody. Left panels, HE staining; right panels, immunohistochemical staining with anti-MLH1 antibody. The type I (with hyperplasia) endometrial cancer (nos. 5, 9, 10), the type II (without hyperplasia) endometrial cancer (nos. 13, 16, 19), T: tumor region, N: normal region, T/N: gene expression of the tumor/gene expression of the normal region determined by cDNA expression arrays (scale bar = 200 μm).
cancer and colorectal cancer (43, 44). STAT1 and STAT2 were reported to be up-regulated in colon cancer and squamous cell carcinoma of the skin (45, 46). In the present study, STAT1 expression was up-regulated in type II, and STAT2 expression was up-regulated in type I endometrial cancer. STATs are important mediators in IFN-α signaling, and STAT12 was reported to be down-regulated in both with hyperplasia and without hyperplasia endometrial cancers (12). Taken together, we attributed the carcinogenesis of endometrial cancers to these alterations in the expression of multiple genes related to cell adhesion, invasion, cell proliferation, transcription, and general metabolism.

As described above, in comparison between two types of cancer, the expression level of each gene in the cancer cells was normalized to that in the corresponding normal cells by calculating the T/N ratio to eliminate biological variations among individual patients. However, we cannot rule out the possibility that the gene expression levels in tumors are the same in both types of cancer, but those in the adjacent normal tissue were affected more by the presence of tumor in one type than the other. Moreover, if the biological variations in gene expression between different individuals are significantly larger than that within a single individual, these genes cannot serve as good markers for endometrial cancers. To observe type-specific gene expression and select good markers for endometrial cancers, not only the T/N ratio but also the absolute levels of gene expression in cancer cells should be considered.

In summary, by using cDNA expression arrays and LCM, we identified genes, some of which were differentially expressed in cancer cells and normal cells, and others between type I cancers with hyperplasia and type II cancers without hyperplasia. Because we used an easy criterion ($P < 0.05$) and picked up a number of candidate discriminating genes between normal and cancer cells and between type I and type II cancers, these genes likely contain a substantial number of false positives. To establish the specificity of each gene, additional studies encompassing a larger number of cases will be needed. Moreover, to demonstrate causal relationships between the expression of these genes and endometrial carcinogenesis, functional analysis will also be needed. However, the study described here and elsewhere (12) will help us to develop novel strategies to diagnose, treat, and ultimately prevent endometrial carcinogenesis, as well as to understand molecular mechanisms of endometrial cancers.

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