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Loss of Heterozygosity on 10q23.3 and Mutation of the Tumor Suppressor Gene *PTEN* in Benign Endometrial Cyst of the Ovary: Possible Sequence Progression from Benign Endometrial Cyst to Endometrioid Carcinoma and Clear Cell Carcinoma of the Ovary[1]

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ABSTRACT

Loss of heterozygosity (LOH) at locus 10q23.3 and mutation of the *PTEN* tumor suppressor gene occur frequently in both endometrial carcinoma and ovarian endometrioid carcinoma. To investigate the potential role of the *PTEN* gene in the carcinogenesis of ovarian endometrioid carcinoma and its related subtype, clear cell carcinoma, we examined 20 ovarian endometrioid carcinomas, 24 clear cell carcinomas, and 34 solitary endometrial cysts of the ovary for LOH at 10q23.3 and point mutations within the entire coding region of the *PTEN* gene. LOH was found in 8 of 19 ovarian endometrioid carcinomas (42.1%), 6 of 22 clear cell carcinomas (27.3%), and 13 of 23 solitary endometrial cysts (56.5%). In 5 endometrioid carcinomas synchronous with endometriosis, 3 cases displayed LOH events common to both the carcinoma and the endometriosis, 1 displayed an LOH event in only the carcinoma, and 1 displayed no LOH events in either lesion. In 7 clear cell carcinomas synchronous with endometriosis, 3 displayed LOH events common to both the carcinoma and the endometriosis, 1 displayed an LOH event in only the carcinoma, and 3 displayed no LOH events in either lesion. In no cases were there LOH events in the endometriosis only. Somatic mutations in the *PTEN* gene were identified in 4 of 20 ovarian endometrioid carcinomas (20.0%), 2 of 24 clear cell carcinomas (8.3%), and 7 of 34 solitary endometrial cysts (20.6%). These results indicate that inactivation of the *PTEN* tumor suppressor gene is an early event in the development of ovarian endometrioid carcinoma and clear cell carcinoma of the ovary.

INTRODUCTION

The tumor suppressor gene *PTEN/MMAC1*,[3] located on chromosome arm 10q (10q23.3), was first reported in 1997 by Li *et al.* (1). Frequent LOH at 10q23.3 and mutations of the gene have been found in various types of cancer (1–5), and germ-line mutations of *PTEN* have also been associated with some familial neoplastic diseases such as Cowden disease, Lhermitte-Ducos disease, and Bannayan-Zonana syndrome (6–8). Introduction of wild-type *PTEN* into the mutant *PTEN* glioma cell line results in growth suppression *in vivo* and *in vitro* (9). *PTEN* encodes a phosphatase that dephosphorylates phosphatidylinositol-3,4,5-triphosphate. The function of the phosphatase is to interfere with the function of phosphatidylinositol-3,4,5-triphosphate, to inhibit cell death mediated by protein kinase B, and to encourage cell proliferation (10, 11). Furthermore, Di Cristofano and Pandolfi (12) reported that loss of function of just a single allele of *PTEN* is sufficient to confer a growth advantage. Their results indicated that *PTEN* mutation without LOH in the *PTEN* region or LOH in the *PTEN* region without mutation can reduce the function of PTEN. Recently, Perren *et al.* (13) examined the expression of the *PTEN* gene in breast cancer immunohistochemically and showed that hemizygous *PTEN* deletions were well correlated with lack of staining for *PTEN* protein.

PTEN gene abnormalities have been identified in various types of human carcinoma, including brain, endometrium, prostate, breast, thyroid, liver, lung (small cell carcinoma), and head and neck carcinomas and lymphomas (1, 2, 5, 14–17). In particular, high frequencies of LOH at 10q23.3 and mutation of the gene have been reported in glioma, endometrial carcinoma of the uterus, and ovarian endometrioid carcinoma (1, 2, 18–20). However, the roles of the *PTEN* gene in the carcinogenesis of glioma and endometrial carcinoma of the uterus seem to be different. Rasheed *et al.* (21), Maier *et al.* (22), and Davis *et al.* (23) reported that the *PTEN* gene mutations are restricted to high-grade rather than low-grade gliomas and may be associated with the transition from a low histological grade to anaplasia. In contrast, Risinger *et al.* (24) reported that, in the genesis of endometrial carcinoma, *PTEN* mutations are associated with early-stage, rather than late and metastatic, carcinomas. Furthermore, Maxwell *et al.* (25) have found frequent *PTEN* mutations in endometrial hyperplasia with and without atypia. These reports indicate that inactivation of the *PTEN* gene is an early event in the development of endometrial carcinoma of the uterus.

Endometriosis, the presence of ectopic endometrial tissue, is a common gynecological disease and is considered to be a benign tumor. Malignant transformation of endometriosis was first documented in 1925 by Sampsons (26) and has been thought to occur in 0.7–1.0% of all cases of endometriosis (27–29). Genetically, ovarian endometrial cysts have a monoclonal origin (30), and endometrioid and clear cell carcinoma of the ovary may arise through malignant transformation of ectopic endometrium (31). These findings support the possibility that endometriosis is a precancerous form of certain types of ovarian cancer. The aim of the present study was to assess the role of LOH at the 10q23.3 locus and *PTEN* gene mutation in the multistep carcinogenesis of ovarian clear cell and endometrioid carcinoma. We examined ovarian endometrial cysts and ovarian endometrioid and clear cell carcinoma for LOH at the loci flanking the *PTEN* gene and mutation of the *PTEN* gene, using a laser-assisted microdissection method (32). We found frequent LOHs at 10q23.3 and mutations of the *PTEN* gene in endometrial cysts and clear cell carcinoma of the ovary, as well as in endometrioid carcinoma of the ovary, suggesting that there is a sequential progression from ovarian endometrial cyst to endometrioid or clear cell carcinoma of the ovary.

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[3] The abbreviations used are: PTEN/MMAC1, phosphatase and tensin homologue deleted on chromosome 10/mutated in multiple advanced cancers 1; LOH, loss of heterozygosity; SSCP, single-strand conformational polymorphism.

MATERIALS AND METHODS

Cases and Microdissection. We examined 20 endometrioid carcinomas, 24 clear cell carcinomas, and 34 solitary endometrial cysts of the ovary, which were resected at the University Hospital of Tsukuba (Ibaraki, Japan) between 1976 and 1998. We also examined 12 specimens of normal endometrium without endometriosis or leiomyoma. We used normal fallopian tubes or lymph nodes without metastases as normal controls for the endometrial cyst and carcinoma cases and myometrium as a normal control for normal endometrial tissue. Eight of the 20 cases of endometrioid carcinoma and 12 of the 24 cases of clear cell carcinoma contained apparently benign ectopic endometrium in the same ovary. All specimens were fixed with 10% formalin and embedded in paraffin. After histological examination, the ectopic endometrial cells, carcinoma cells, normal endometrium, and normal cells that were used as normal controls were microdissected with a Pixcell Laser Captured Microdissection System (Arcturus Engineering, Inc., Mountain View, CA; Fig. 1). Finally, we microdissected about 20–40 cells from each specimen and extracted the genomic DNA.

LOH Analysis. Three microsatellite markers (*D10S215*, *D10S541*, and *D10S608*) were used to evaluate LOH on 10q23.3. All primers used in this study were obtained from Research Genetics (Huntsville, AL). Genomic DNA corresponding to DNA extracted from eight cells was subjected to PCR amplification in 10 μl of reaction mixture. The reaction mixture consisted of 3.6 units of Ex-Taq DNA polymerase (Takara, Tokyo, Japan); 200 μM dATP, dTTP, and dGTP; 20 μM dCTP; 5 μCi of [α-^{32}P]dCTP (Amersham Life Science, Buckinghamshire, United Kingdom); 33.5 mM Tris-HCl (pH 8.8); 1.5 mM $MgCl_2$; 16 mM $(NH4)_2SO_4$; 0.01% Tween 20; and 0.3 μl of each primer as supplied (20 μM each). PCR was carried out over 35 amplification cycles for 45 s at 94°C, 45 s at 55°C, and 60 s at 72°C in a Takara Thermal Cycler MP (Takara). The PCR products were resolved on a 6% denaturing polyacrylamide gel and visualized by autoradiography film (Kodak, Rochester, NY) exposure.

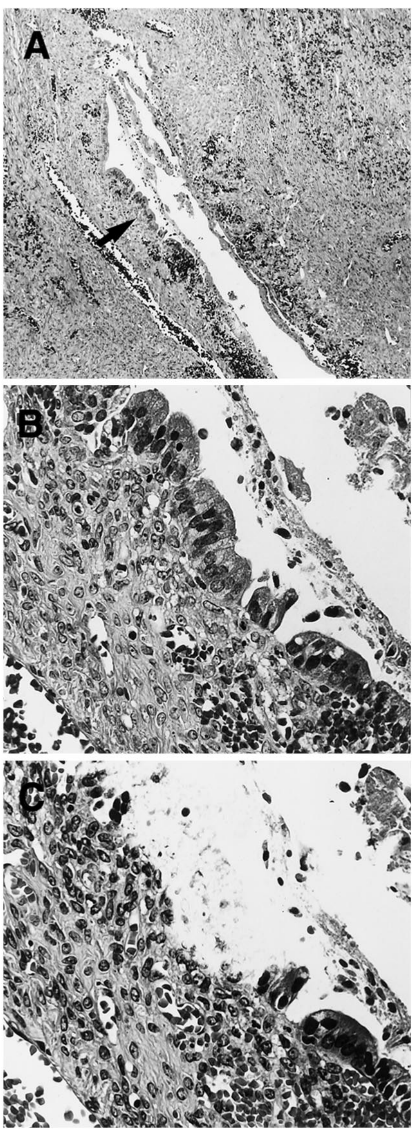

Fig. 1. Histology of endometrial cyst (case 6); H&E, ×40 (*A*). *Arrow*, microdissected area. High-power view of the area before (*B*) and after (*C*) microdissection (×400).

SSCP Analysis. Nine exons of *PTEN* (except for the first primer set of exon 5) were amplified separately using the primer sets described by Risinger *et al.* (19). We used ATCTTTTTACCACAGTTGCAC and GTCCCTTTC-CAGCTTTACAG as the first primer set for exon 5. Genomic DNA corresponding to DNA extracted from eight cells was subjected to PCR amplification in 10 μl of the reaction mixture used in the LOH analysis. PCR was carried out as described previously (19). The PCR products were resolved on a 0.5× Mutation Detection Enhancement gel (FMC Bioproducts, Rockland, ME) and visualized by autoradiography film (Kodak, Rochester, NY) exposure.

DNA Sequencing. Shifted SSCP bands were excised from the Mutation Detection Enhancement gel. We extracted the DNA from the gel with distilled water and reamplified it using the original PCR primers. The reamplified PCR products were cloned into the pCRII TA vector (Invitrogen, San Diego, CA), according to the company's instructions, and then sequenced with an ABI PRISM 310 Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer, Foster City, CA). For the cases with mutations, we repeated the microdissection, SSCP analysis and sequencing, and confirmed the results.

RESULTS

LOH Analysis. We examined the genotypes of 20 endometrioid carcinomas of the ovary at three highly polymorphic loci distributed at 10q23.3 (*D10S215*, *D10S541*, and *D10S608*). As Table 1 shows, 8 of 19 informative cases of endometrioid carcinoma (42.1%) demonstrated LOH in the 10q23.3 region. To examine whether the LOH found frequently in this region in endometrioid carcinoma of the ovary was also present in clear cell carcinoma and endometrial cysts (which are thought to be associated histologically with endometrioid carcinoma of the ovary), we further studied 24 clear cell carcinomas and 34 endometrial cysts of the ovary (Table 1 and Fig. 1). Six of 22 informative cases of clear cell carcinoma (27.3%) and 13 of 23 informative cases of endometrial cyst (56.5%) demonstrated LOH at 10q23.3. None of the 12 specimens of normal endometrium showed LOH at *D10S215*, *D10S541*, or *D10S608*.

SSCP and Sequencing. SSCP analysis of the *PTEN* gene was performed on 20 endometrioid carcinomas, 24 clear cell carcinomas, and 34 solitary endometrial cysts without carcinoma. We screened the entire *PTEN* coding region (9 exons; Fig. 2). Four, 3, and 11 abnormally shifted bands that were detected from cases of ovarian endometrioid carcinoma, clear cell carcinoma, and endometrial cyst, respectively, were eluted from the gel, and the DNA extracted was sequenced (Table 2). There was one missense mutation (transversion), two nonsense mutations, and one deletion in four endometrioid carcinomas. Two missense mutations (transitions) and one deletion were detected in two clear cell carcinomas. Eight missense mutations (seven transitions and one transversion) and two deletions were detected in seven endometrial cysts. In the ovarian endometrioid carcinomas, the codons that showed the mutation were scattered between exons 1 and 9, but they appeared to cluster around the catalytic signature motif of the *PTEN* gene in the clear cell carcinomas and endometrial cysts (Fig. 3). Two of four cases of endometrioid carcinoma, one of two cases of clear cell carcinoma, and five of eight cases of solitary endometrial cyst were accompanied by LOH at 10q23.3.

Comparison of LOH at 10q23.3 in Ovarian Carcinoma and Synchronous Apparently Benign Endometrium. We detected apparently benign ectopic endometrium synchronously in 8 of 20 endo-

Table 1 *Frequency of LOH at 10q23.3 in normal endometrium, endometrial cyst, endometrioid carcinoma, and clear cell carcinoma of the ovary*

	Allelic loss/informative specimen (%)			
	Normal[a]	Cyst	Em	CCC
D10S215	0/4 (0%)	3/11 (27.3%)	6/15 (40.0%)	2/20 (10.0%)
D10S541	0/7 (0%)	8/14 (57.1%)	3/5 (60.0%)	3/9 (33.3%)
D10S608	0/12 (0%)	6/12 (50.0%)	3/16 (18.8%)	4/15 (26.7%)
Total	0/12 (0%)	13/23 (56.5%)	8/19 (42.1%)	6/22 (27.3%)

[a] Normal, normal endometrium; Cyst, solitary endometrial cyst; Em, endometrioid carcinoma; CCC, clear cell carcinoma.

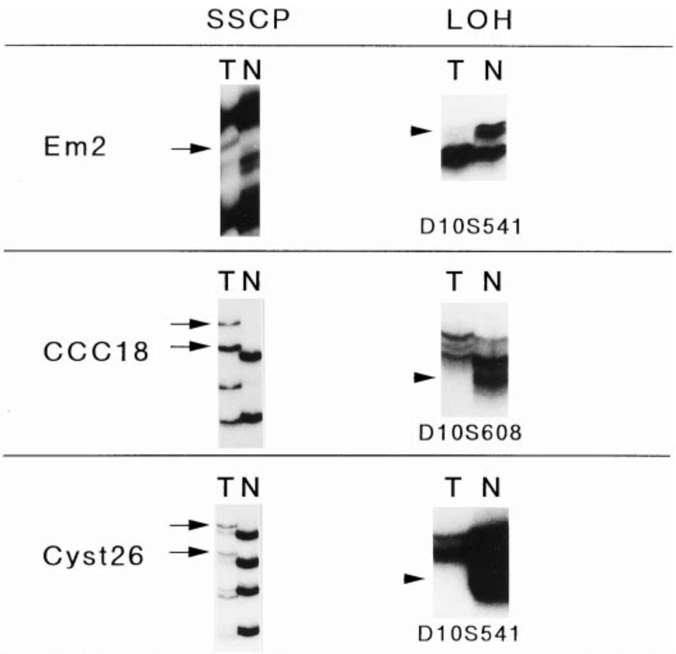

Fig. 2. Representative results of LOH at 10q23.3 and SSCP analysis of *PTEN* gene in endometrial cyst (case 26), endometrioid carcinoma (case 2), and clear cell carcinoma (case 18) of the ovary. *Arrow*, shifted band in SSCP analysis. *Arrowhead*, allelic loss. *T*, tumor; *N*, normal.

metrioid carcinomas and 12 of 24 clear cell carcinomas. These endometrial tissues were diagnosed as benign ectopic endometrium histologically and were also examined for LOH in the *PTEN* region (*D10S215* and *D10S541*), the patterns of LOH being compared between them and the carcinomas. Five of eight endometrioid carcinoma cases synchronous with endometriosis and 7 of 12 clear cell carcinoma cases synchronous with endometriosis were informative. Of these five endometrioid carcinoma cases, three displayed LOH events common to both the carcinoma and endometriosis, one displayed an LOH event only in the carcinoma, and one displayed no LOH event in either lesion (Fig. 4). Of the seven clear cell carcinoma cases, three displayed LOH events common to both the carcinoma and endometriosis, 1 displayed an LOH event only in the carcinoma, and three displayed no LOH event in either lesion. In no case was LOH detected only in endometriosis.

DISCUSSION

In this study, we confirmed the high frequency of LOH at 10q23.3 in endometrioid carcinoma of the ovary (42.1%) and demonstrated high frequencies of LOH at 10q23.3 in solitary endometrial cysts (56.5%) and clear cell carcinoma of the ovary (27.3%). SSCP and DNA sequence analysis also displayed somatic mutations in the solitary endometrial cysts (20.6%), as well as in the endometrioid type (20.0%) and clear cell type (8.3%) of ovarian carcinoma.

Clinicopathologically, endometrial cysts of the ovary are thought to develop from ectopic endometrium in the ovary, and they have recently been confirmed to grow monoclonally (30). Jiang *et al.* (33) found that <20% of endometrial cysts had LOH at the loci (chromosomes 9q, 11q, and 22q) of candidate tumor suppressor genes associated with ovarian cancers. We found more frequent LOH at 10q23.3 in endometrial cysts of the ovary than at these other loci (see Table 1). Furthermore, we observed mutations of the *PTEN* gene in seven cases of solitary endometrial cyst (20.6%), and five of these seven were accompanied by LOH at 10q23.3 (Table 2). Maxwell *et al.* (25) reported somatic mutation of the *PTEN* gene in ~20% of cases of endometrial hyperplasia, which is thought to be the precursor of endometrial carcinoma of the uterus, and the frequencies did not differ between hyperplasia with atypia and that without atypia. These data indicated the genetic sequence from endometrial hyperplasia to endometrial carcinoma of the uterus. The high frequency of *PTEN* gene mutation we observed in solitary endometrial cysts suggests a similar genetic association between solitary endometrial cyst of the ovary and ovarian endometrioid carcinoma. Our results indicate that LOH at 10q23.3 and mutations of the *PTEN* gene are very early events in the development of ovarian endometrioid carcinoma and also support the concept that solitary endometrial cysts of the ovary are a precancerous form of ovarian endometrioid carcinoma. In contrast, the frequency of *PTEN* gene alterations (including LOH on 10q23.3 and somatic mutations of the *PTEN* gene) in other types of ovarian carcinoma, such as serous or mucinous adenocarcinoma, has been reported to be very small compared with that in ovarian endometrioid carcinoma (20).

Table 2 *PTEN mutation and LOH at 10q23.3 in endometrial cyst, endometrioid carcinoma, and clear cell carcinoma*

Case[a]	Exon	Base change	Predicted effect	LOH on 10q
Em1	2	80–90del	Y27X	Not LOH
Em9	2	160G→T	R54L	LOH
Em2	7	697C→T	R233X	LOH
Em10	7	697C→T	R233X	Not LOH
CCC4	5	451G→A	A151T	Not LOH
CCC18	6	[498delA;568C→T]	L182X	LOH
Cyst8	1	7G→T	A3S	Not LOH
Cyst7	5	451G→A	A151T	LOH
Cyst11	5	451G→A	A151T	ND
Cyst31	5	451G→A	A151T	LOH
Cyst34	5	451G→A	A151T	LOH
Cyst8	6	498delA;568C→T	L182X	Not LOH
Cyst26	6	[498delA;568C→T]	L182X	LOH
Cyst28	8	940G→A	E314K	LOH

[a] Em, endometrioid carcinoma; CCC, clear cell carcinoma; Cyst, solitary endometrial cyst; ND, not determined; Not LOH, without LOH at *D10S215, D10S541*, or *D10S608*; LOH, with any LOH at *D10S215, D10S541,* or *D10S608.*

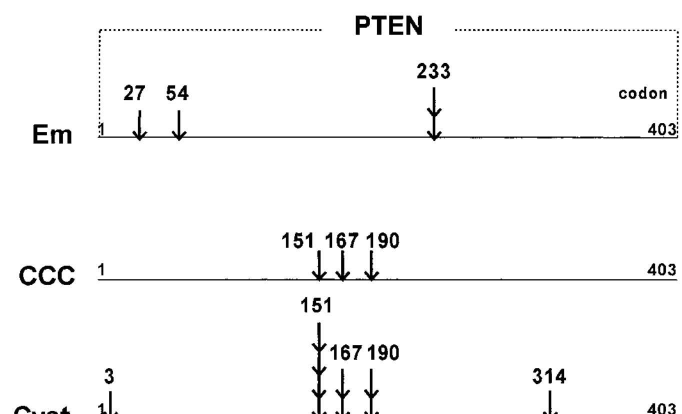

Fig. 3. Map of mutation distribution in the *PTEN* gene. Each *arrow* represents one case of *PTEN* mutation.

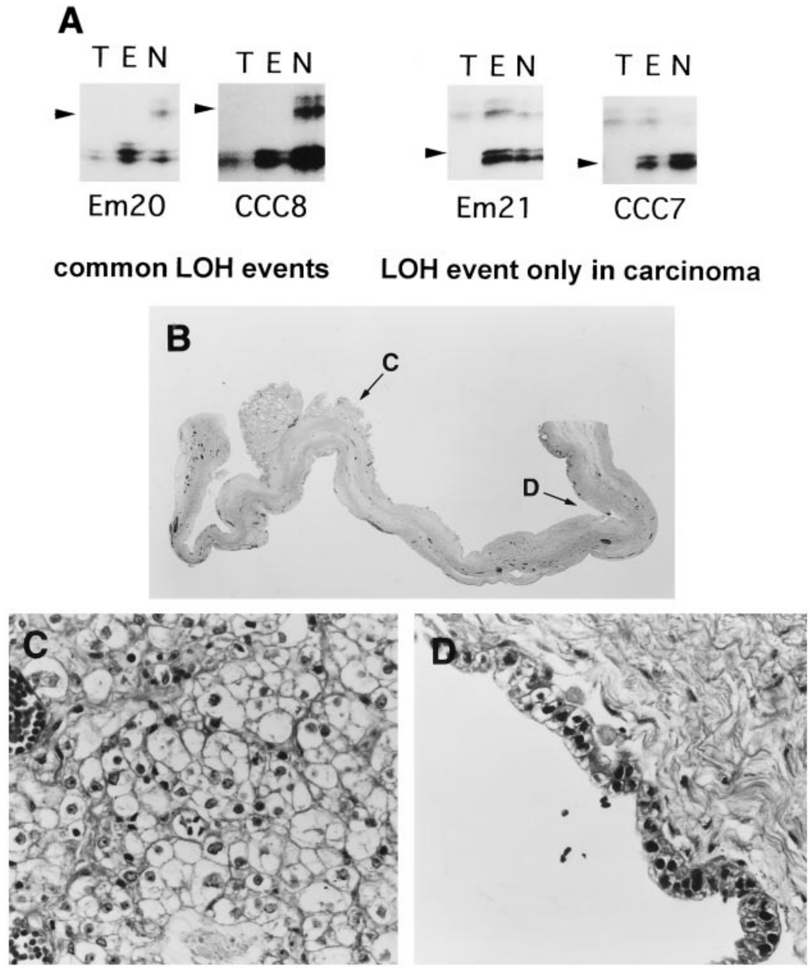

Fig. 4. Comparison of LOH at 10q23.3 in ovarian carcinoma and synchronous endometrial cyst. LOH analysis at *D10S541* in each lesion of endometrioid carcinoma, cases 20 and 21 (*Em20* and *Em21*), and clear cell carcinoma, cases 7 and 8 (*CCC7* and *CCC8*; *A*). *T,* carcinoma; *E,* benign ectopic endometrium; *N,* normal; *arrowhead,* allelic loss. *B,* histological section of clear cell carcinoma case 7. High-power views of clear cell carcinoma (*C*) and endometrial cyst (*D*) are shown; H&E, ×200.

However, the frequency and significance of *PTEN* gene abnormalities in clear cell carcinoma of the ovary is still unclear. Obata *et al.* (20) reported LOH at 10q23.3 in one of seven informative cases of clear cell carcinoma but found no mutations of the *PTEN* gene in any of the cases they examined (0 of 8). We examined a large number of clear cell carcinomas (24 cases) and demonstrated LOH at 10q23.3 in 6 of 22 informative cases (27.3%); we found *PTEN* mutations in 8.3% (2 of 24). Although LOH at 10q23.3 and *PTEN* mutation occurred less frequently in clear cell carcinoma than in endometrioid carcinoma, there were no significant differences in their occurrences in the two types of carcinoma, and the frequency of LOH was still higher than in other histological types of ovarian carcinoma.

The mutation spectra of the *PTEN* gene in these three different types of ovarian tumors were of interest. Mutations in the endometrial cysts and clear cell carcinomas were concentrated at exons 5–6, which encode the phosphatase domain of the *PTEN* gene (Fig. 3). These results indicate the functional similarity of tumorigenesis between endometrial cyst and clear cell carcinoma of the ovary.

To confirm the sequences of carcinogenesis between endometrial cyst and ovarian endometrioid carcinoma or clear cell carcinoma of the ovary, we investigated LOH events at *D10S215* and *D10S541* (which flank the *PTEN* gene) in five endometrioid carcinomas and seven clear cell carcinomas that were thought to have occurred synchronously with endometrial cysts. We found cases that displayed LOH events in both the carcinoma and apparently benign cyst, or only in the carcinoma, but none of the cases showed LOH events only in the cyst (Fig. 4). Although there is a possibility that cystic lesions may be part of an extremely well differentiated adenocarcinoma, these results are thought to support the concept of sequential progression from endometrial cyst of the ovary to ovarian endometrioid or clear cell carcinoma.

In this study, we used a laser-assisted microdissection system (32), which enabled us to collect not only tumor cells from carcinoma tissue but also the epithelial lining cells of endometriosis tissue, without contamination by nontumor cells such as lymphocytes, endothelial cells, and fibroblasts. This approach made it possible to analyze various genetic alterations in the endometrial cysts. We expect that this technique will be very useful for investigating genetic alterations in other tissues or precancerous lesions such as ulcerative colitis, lung fibrosis, or benign prostatic hyperplasia, because in these lesions, the dysplastic or atypical cells that are thought to be carcinoma precursors are widely mixed with numerous nontumor cells. Early genetic alterations in various precancerous cells detected by light microscopy can be readily identified by the tissue-microdissection method.

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