Allelic loss studies do not provide evidence for the "endometriosis-as-tumor" theory

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Objective: To identify consistent genetic changes in endometriosis samples to determine whether endometriosis lesions are true neoplasms.
Design: We analyzed ovarian endometriosis lesions for loss of heterozygosity (LOH) at 12 loci of potential importance (D9S1870, D9S265, D9S270, D9S161, D11S29, D1S199, D8S261, APOA2, PTCH, TP53, D10S541, and D10S1765), including some at which genetic changes were previously reported in endometriosis.
Setting: Molecular biology laboratory in a university hospital department.
Patient(s): Seventeen women with ovarian endometriosis.
Intervention(s): Laser capture microdissection to separate the endometriotic epithelium, the adjacent endometriotic stroma, and surrounding normal ovarian stromal tissue, followed by DNA extraction and polymerase chain reaction amplification of polymorphic microsatellite markers.
Main Outcome Measure(s): Fluorescence-based quantitation for the LOH analysis.
Result(s): We identified LOH in only one lesion at one locus (D8S261).
Conclusion(s): Our data do not support the hypothesis that ovarian endometriosis is a true neoplasm. (Fertil Steril® 2005;83(Suppl 1):1134–43. ©2005 by American Society for Reproductive Medicine.)

Key Words: Endometriosis, loss of heterozygosity, ovarian carcinoma, laser capture microdissection

Endometriosis, defined as the presence of endometrial glands and stroma outside the uterus, can cause debilitating pelvic pain, painful periods, and subfertility and is believed to affect millions of women worldwide. The frequency of endometriosis has been difficult to determine because the symptoms are variable and diagnosis requires visual inspection of the pelvis at surgery. However, it is known that chronic pelvic pain, one of the main symptoms of endometriosis, is extremely common (1, 2).

Despite its clinical importance, little is known about the molecular defects that cause the disease, although endometriosis is thought to be a complex trait, resulting from the interaction between multiple genetic loci and a variety of environmental risk factors (3). Delineating the molecular mechanisms involved in the development of endometriosis may therefore lead to improved ways of managing the disease and would facilitate the rational design of novel drugs.

Although endometriosis is a "benign" disease, it has many characteristics of neoplasia (used in this context to mean clonal proliferation of cells arising from genetic changes that provide the clone with a replicative advantage). Endometriosis may show unrestrained growth and increased vascularization and even features classically associated with malignancy, such as tissue invasion and metastasis (4). There is also evidence that endometriosis is associated with, and might be a precursor to, endometrioid and clear cell ovarian carcinoma (4). It is therefore believed that the genetic alterations involved in both tumorigenesis and the development of endometriosis could overlap and that identifying the genes involved in endometriosis might give critical clues to the initiation of endometrioid and clear cell ovarian carcinomas.

Previous studies have analyzed ovarian endometriotic cysts for clonal origins and for genetic alterations (5–8). Most have shown ovarian lesions to be monoclonal in origin, but this is not necessarily indicative of neoplasia since an endometriotic cyst could simply be a malformation that has developed within a single, stem cell "patch." Conversely, there are reports of polyclonality in endometriosis, which suggest non-neoplastic origins (8–10). The methods used to assess clonality are often unreliable; separating endometriotic from normal tissue was technically difficult before laser capture microdissection became available, and some methods, such as the human androgen receptor assay, tend to produce false negatives because of restriction endonuclease failure. Recently, Mayr et al. (8) used a phosphoglycerate

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kinase-1 polymorphism to analyze ovarian endometriotic cysts for clonal origins by laser microdissection. From the 13 of 29 informative patients, 32 samples were studied and only 2 of 32 were consistent with monoclonal origin.

We believe that demonstrating consistent genetic changes in endometriosis would be the best indication that these lesions are true neoplasms. Several workers have addressed this issue, mostly by testing for allelic loss (loss of heterozygosity [LOH]), and we critically review these studies in our Discussion below. Before doing so, we report our own results from screening 17 samples of ovarian endometriosis for LOH at 12 loci of potential importance in the pathogenesis of endometriosis.

MATERIALS AND METHODS

Anonymized tissue blocks from 17 women with ovarian endometriosis were identified from the histopathology archives of the John Radcliffe Hospital, Oxford. Institutional Review Board approval from our local ethics committee gave us permission to access unlinked, anonymized samples. Hematoxylin and eosin (H&E)-stained slides were reviewed by a single histopathologist (S.M.) to confirm the diagnosis. Ten sections (10 μm) were cut from each lesion, and the sections were lightly stained with H&E. Each section was microdissected using a laser capture microscope (Arcturus Engineering, Mountain View, CA) to separate the endometriotic epithelium, the adjacent stroma, and surrounding normal tissue.

Extraction of DNA was performed by incubating the samples in 20–250 μL 1 $\times$ polymerase chain reaction (PCR) buffer (Promega UK, Southampton, UK), pH 8, with 200 μg/mg proteinase K at 56°C overnight. Polymorphic microsatellite markers were chosen that map to chromosomal regions containing potentially important genes involved in tumorigenesis (D9S1870, D9S265, D9S270, D9S161, D11S29, D1S199, D8S261, APOA2, PTCH, TP53, D10S541, and D10S1765 [http://genome.ucsc.edu/]) (Table 1).

One oligonucleotide from each pair was fluorescently labeled (with FAM, HEX, or NED (Applied Biosystems, Cheshire, UK). PCR was performed in a 30-μL reaction containing 1.5 mM Mg^{2+}, 500 μM dNTPs, and 0.75–1.0 μM concentrations of each primer pair, 0.75 unit Taq polymerase (Promega), and 1 μL of the extracted DNA (from endometriotic epithelium, adjacent endometriotic stroma, or normal ovarian stromal tissue). PCR amplification was per-

TABLE 1

Microsatellite markers.

Locus	Chromosomal location	Primer sequence
D9S1870	9p21.3	TGGGTATGGTTTTCTGG
		TTGAGGCAGGTCAAATAA
D9S265	9p21.3	TGGTGAAGCCTATTCTTGGT
		CATTGGCAAAGTGTGCG
D9S270	9p21.1	AGGTGTAGTCCTTCTGGAATTT
		GATGTGACTGCTGTTAAAACTAGAG
D9S161	9p21.2	TGCTGCATAACAAATTACCAC
		CATGCCTAGACTCCTGATCC
D11S29	11q23.3	TCTAGCTCCACCATCCTGTG
		ACAACACACTGCCACAAGAC
D1S199	1p36.13	GGTGACAGAGTGAGACCCTG
		CAAAGACCATGTGCTCCGTA
D8S261	8p22	TGCCACTGTCTTGAAAATCC
		TATGGCCCAGCAATGTGTAT
APOA2	1q23.3	CTTGAGCTATTTCGTGGAACTTGG
		CATTCAGCATTTATTGTAGCAAAGAG
PTCH	9q22.3	CGCATAGCGTGTTTGAAAGTT
		GTATTTGTGCATTGGGCTCC
TP53	17p13.1	AGGGATACTATTCAGCCCGAGGTG
		ACTGCCACTCCTTGCCCCATTC
D10S541	10q23.31	AAGCAAGTGAAGTCTTAGAACCACC
		CCACAAGTAACAGAAAGCCTGTCTC
D10S1765	10q23.31	ACACTTACATAGTGCTTTCTGCG
		CAGCCTCCCAAAGTTGC

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formed as follows: 96°C for 5 minutes, 40 cycles of 96°C for 30 seconds, an annealing step of between 50°C and 55°C for 30 seconds, 72°C for 30 seconds, followed by a final extension step of 72°C for 10 minutes in an Eppendorf PCR machine. The PCR products were run on an ABI 3700 Genetic Analyzer, and microsatellites were analyzed for allelic imbalance using the Genotyper program (Applied Biosystems).

In informative (heterozygous) cases, allelic loss at each marker was scored in the endometriotic epithelium or the stroma if the area under one allelic peak was reduced by >50% relative to the other allele, after correcting for the relative peak areas in DNA from the normal tissue.

RESULTS

To determine whether endometriotic lesions are true neoplasms we sought to identify consistent genetic alterations by performing LOH analysis on 17 archival ovarian endometriosis samples (Table 2). Each sample was microdissected using a laser capture microscope to separate the endometriotic epithelium, the adjacent stroma, and surrounding normal tissue before DNA extraction and LOH analysis.

For LOH analysis, 12 polymorphic microsatellite markers were chosen that map to chromosomal regions containing potentially important genes involved in tumorigenesis (D9S1870, D9S265, D9S270, D9S161, D11S29, D1S199, D8S261, APOA2, PTCH, TP53, D10S541, and D10S1765). Markers around $p16^{INK4a}$ (D9S265, D9S1870, D9S270, and D9S161) and TP53 were chosen because these tumor suppressors are commonly mutated in multiple cancer types (11, 12) and LOH has been detected at these loci in endometriosis samples (10, 13, 14). In addition, a TP53 mutation was found in 1/14 cases of endometriosis (14) and abnormal methylation of $p16^{INK4a}$ was detected in 1/46 endometriosis samples (15). PTCH is also a tumor suppressor that is mutated in basal cell carcinomas, medulloblastomas, and other cancers (16). D10S541 and D10S1765 map to the PTEN locus; PTEN is a tumor suppressor gene that is mutated in a variety of sporadic cancers and in two autosomal dominant hamartoma syndromes (17). In addition, inactivation of PTEN has been reported as an early event in the development of ovarian endometrioid and endometrial carcinomas (18, 19). D11S29, D1S199, and D8S261 map to regions that commonly show LOH in many tumor types, including ovarian carcinomas (20–24). APOA2 maps to 1q21-q23, and LOH has previously been detected at this locus in an endometriosis sample (13).

We found that LOH at all the sites analyzed was very uncommon in both endometriotic epithelium and stroma. In the overwhelming majority of lesions, LOH was unequivocally absent (Table 3; examples are shown in Fig. 1), and the allelic ratios in the endometriotic samples were generally very similar to those in the normal tissue, providing no evidence to suggest that allelic loss had been masked by contaminating normal tissue. Just one lesion showed LOH, and this was at just one microsatellite marker (D8S261) (Fig. 2). Interestingly, both the epithelial and stromal parts of this lesion showed the change, suggesting that they may be derived from the same clone. Our data have provided no evidence to suggest that ovarian endometriosis is a true neoplasm.

TABLE 2

Clinical data of ovarian endometriosis samples.

Case	Age (years)	Diagnosis
1	44	Endometriotic foci (stage 4; no atypia)
2	44	Endometriotic cyst and foci (no atypia)
3	33	Endometriotic cyst and foci (no atypia)
4	40	Endometriotic foci (no atypia)
5	28	Endometriotic cyst (no atypia)
6	21	Endometriotic cysts (no atypia)
7	43	Endometriotic cyst (no atypia)
8	31	Endometriotic cyst (no atypia)
9	31	Endometriotic cyst and foci (no atypia)
10	46	Endometriotic cyst (no atypia)
11	46	Endometriotic cyst and foci (no atypia)
12	51	Endometriotic foci (no atypia)
13	27	Endometriotic cyst and foci (no atypia)
14	27	Endometriotic foci (no atypia)
15	48	Endometriotic foci (no atypia)
16	33	Endometriotic cyst (no atypia)
17	33	Endometriotic cyst (no atypia)

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DISCUSSION

Our analyses using fine-scale laser microdissection and fluorescence-based quantitation of allele dosage showed that LOH at all the sites analyzed was very uncommon in both endometriotic epithelium and stroma. Just one lesion showed LOH, and this was at just one microsatellite marker (D8S261). Interestingly, both the epithelial and stromal parts of this lesion showed the change, which suggests that they may be derived from the same clone. In general, however, our data provided no evidence to suggest that ovarian endometriosis is a true neoplasm.

Some of our microsatellite markers were chosen because other workers had shown them to undergo allelic loss in endometriosis (summarized in Table 4). Our data therefore differ from those derived by many other investigators. In addition to

TABLE 3

Loss of heterozygosity (LOH) analysis of 17 cases of endometriosis at 12 loci.

Case	D11S29	D1S199	D8S261	D9S187	D9S265	D9S270	D9S161	PTCH	APOA2	TP53	D10S541	D10S1765
1	H	–	H	NI	H	H	NI	H	H	H	NI	H
2	H	H	H	NI	H	H	H	H	H	H	H	–
3	H	NI	H	H	H	H	H	H	H	H	NI	H
4	H	NI	H	H	H	H	H	H	H	H	H	H
5	H	–	H	–	–	H	H	H	NI	H	–	–
6	H	NI	–	NI	H	H	H	H	H	H	H	H
7	H	–	H	NI	H	H	H	H	H	H	H	H
8	H	–	H	H	H	H	H	H	H	H	H	NI
9	H	–	H	H	H	H	H	H	H	H	NI	H
10	H	H	LOH	H	NI	H	H	H	–	H	H	–
11	H	H	H	H	H	H	H	H	H	H	H	H
12	H	H	H	H	H	H	H	H	H	H	H	H
13	H	NI	H	–	–	H	H	H	–	H	NI	NI
14	H	NI	H	H	H	H	H	H	H	H	H	–
15	H	–	H	H	H	H	H	H	H	H	H	NI
16	H	–	NI	H	H	H	H	H	–	H	H	NI
17	NI	NI	H	–	–	H	H	H	–	–	H	–
LOH(%)	0/16	0/4	1/14 (7)	0/10	0/13	0/17	0/16	0/17	0/12	0/16	0/12	0/8

Note: H = retention of heterozygosity; NI = noninformative; – = failed.

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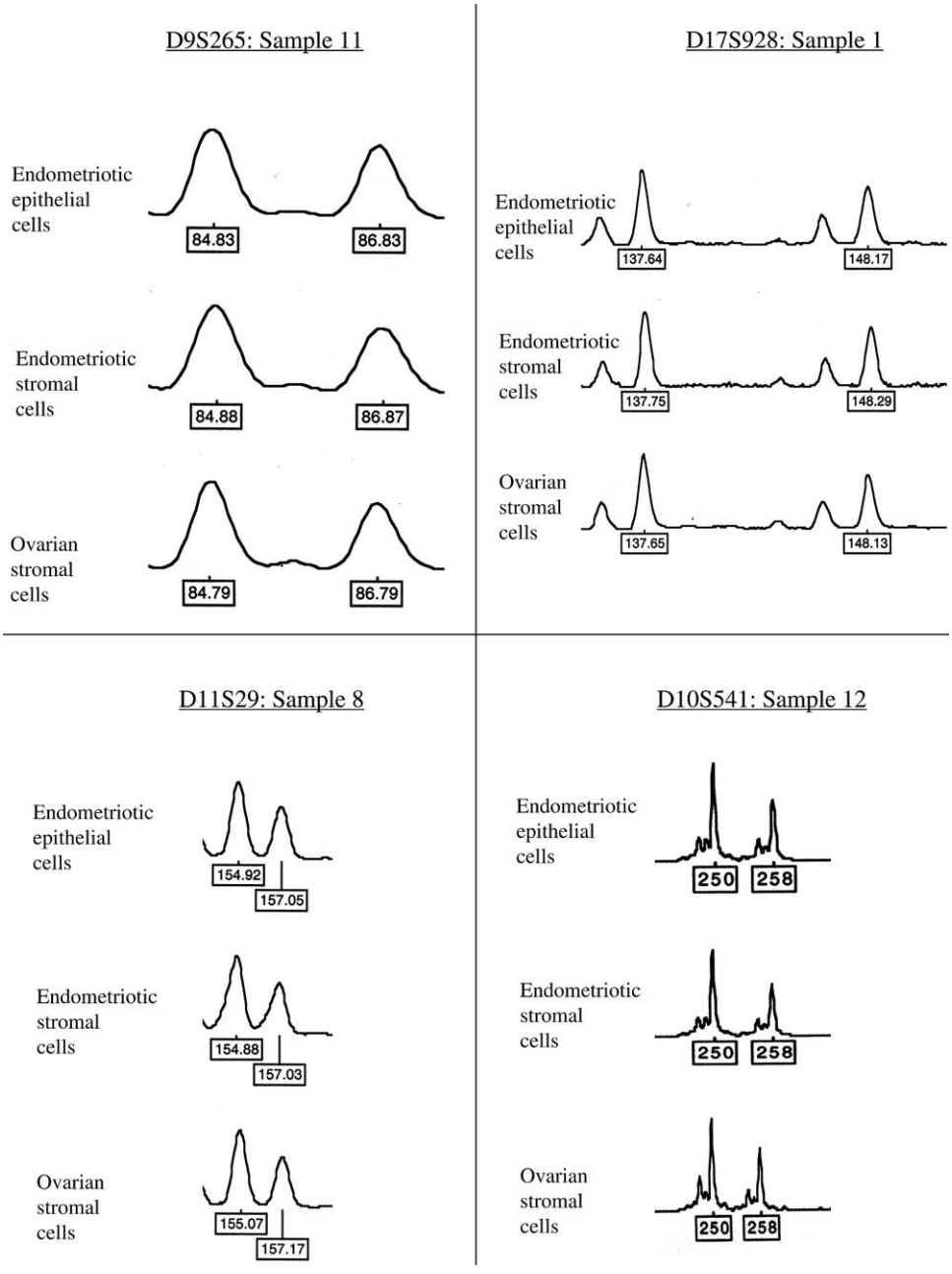
FIGURE 1

Retention of heterozygosity at markers D9S265, D17S928, D11S29, and D10S541 in ovarian endometriotic lesions from samples 11, 1, 8, and 12, respectively. Representative electropherograms from the analysis of DNA extracted from endometriotic epithelium, endometriotic stroma, and adjacent normal ovarian stroma from samples 11, 1, 8, and 12, respectively, are shown. Sizes of the alleles, in base pairs, are indicated under the allele peak.



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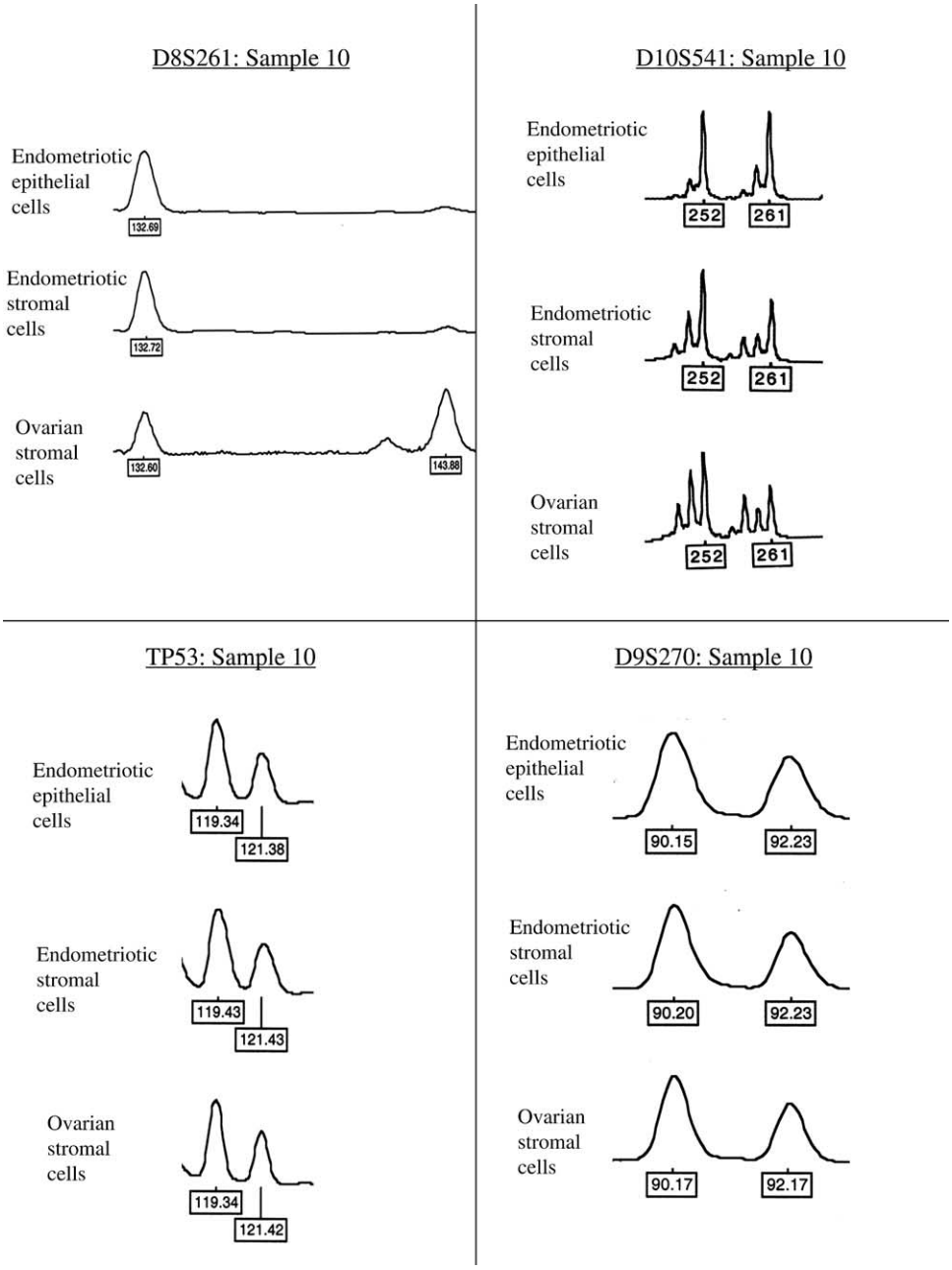
a natural bias in reporting "positive" data, there are several methodological factors that might explain the differences. Other studies have used a variety of methods to score LOH, but only one other study has used fluorescence-based technology: Nakayama et al. detected LOH, at the PTCH locus, in only one of four samples analyzed at seven markers (25). Very few investigators have scored LOH using a quantitative method, and most relied on visual inspection of allelic dosage on autoradiographs or stained agarose gels. Studies might also have suffered from allelic dropout, a phenomenon resulting from random or

FIGURE 2

Lesion 10: lost of heterozygosity at D8S261, but retention of heterozygosity at D10S541, TP53, and D9S270. Representative electropherograms from the analysis of DNA extracted from endometriotic epithelium, endometriotic stroma, and adjacent normal ovarian stroma from sample 10 are shown. Sizes of the alleles, in base pairs, are indicated under the allele peak.



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systematic differences in PCR amplification of different microsatellite alleles. Fluorescence in situ hybridization–based studies that showed gain and loss of genetic material in endometriosis rarely used normal control tissues, and it is possible that some apparent changes resulted from differential probe activity in tissue sections.

Our failure to find allelic loss at the PTEN locus in endometriosis is particularly surprising since in one study LOH at PTEN was detected at high frequency (50%) in endometriotic lesions synchronous with clear cell or endometrioid carcinomas (19). In addition, LOH and missense PTEN mutations were identified in solitary endometrial cysts

TABLE 4

Summary of previous studies analyzing the genetics of endometriosis by loss of heterozygosity (LOH) analysis, fluorescence in situ hybridization (FISH), or CGH (comparative genomic hybridization).

Analysis/ reference	Chromosomes analyzed	No. of samples	Dissection	Scoring of LOH	Results
LOH (10, 14)	2q, 4q, 5p, 6q, 7p, 9p, 11q, 17p, 17q, 22q, Xq	54 endometriosis	Manual	By eye; autoradiograph	LOH detected in 23/54 samples at one or more loci, including 4q (2%), 5q (10%), 6q (7%), 9p (21%), 11q (19%), 22q (19%)
LOH (13)	1p, 1q, 2p, 2q, 3p, 9p, 9q, 17p, 17q	22 endometriosis	Manual	By eye; silver staining	LOH detected in 8/22 (36.4%) samples in at least one locus, including 9p21 (27.3%), 1q21 (4.5%), 17p13.1 (4.5%)
LOH (19)	10q23.3 (PTEN)	23 solitary endometrial cysts, 5 endometriosis with endometrioid carcinoma, 7 endometriosis with clear cell ovarian carcinoma	LCM	By eye; autoradiography	LOH detected in 13/23 cysts, 3/5 endometriosis with endometrioid carcinoma, 3/7 endometriosis with clear cell ovarian carcinoma
LOH (25)	8 markers located around p16, PTCH, WT1, RB1, D14S267, NM23, DPC4, DCC	3 ovarian endometriotic cysts, 1 peritoneal implant	Manual	Florescence based using automated sequencer	LOH detected at *PTCH* in one sample
LOH (30)	17p13.1, 17q22-24, 17q11.2-q12	15 endometriosis samples	No	By eye; autoradiography or ethidium bromide staining	LOH detected in 1/13 at 17q11.2-q12 and 3/13 at 17q22-q24
FISH (30)	Chromosome 17 centromere and p53 locus	14 endometriosis samples	–	–	Monosomy of chromosome 17 centromere and *TP53* detected in some cells in all 14 samples. Some cells had *TP53* loss only

TABLE 4

Continued.

Analysis/ reference	Chromosomes analyzed	No. of samples	Dissection	Scoring of LOH	Results
FISH (31)	Chromosomes 7, 8, 11, 12, 16, 17, and 18.	4 endometriosis samples	–	–	Increased number of cells with [1] monosomy 17 and 16 detected in 1/4; [2] monosomy 16 alone detected in 1/4; and [3] trisomy 11 detected in 1/4
FISH (32)	Chromosome 17	8 endometriosis samples	–	–	Increase in number of cells with chromosome 17 aneuploidy in all samples
CGH (33)	–	18 endometriosis samples	Manual	–	Losses detected in 15/18 samples, including at chromosomes 1p (50%), 22q (50%), 5p (33%), 6q (27%), 7p (22%), 9q (22%), and 16 (22%). Gain detected at 6q, 7q, and 17q in 3 cases
CGH (34)	–	1 cell line and original endometriosis sample	Manual	–	In cell line; gain of chromosomes 1, 2, 3, 5, 6p, 7, 16, 17q, 20, 21q, and 22q; loss of chromosomes 6p, 9, 11p, 12, 13q, 18, and X. Original; gain of 1p, 22q, and X.; loss of 1p, 22q, and chromosome X

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of the ovary. Immunohistochemistry analysis has shown reduced PTEN expression in 7 of 46 (15.21%) endometriosis samples, all of which were removed from women with severe disease (15). However, absent expression was not detected and the PTEN gene was not analyzed for genetic alterations.

It is possible that the endometriotic cysts in our series were earlier-stage lesions than those studied by other groups. Late-stage lesions might gain a neoplastic component and/or tend to acquire more background genetic changes. We suspect that our finding of LOH at D8S261 in one lesion to be such a background change. In keeping with this suggestion, it is notable that studies of endometriosis have shown few consistent patterns of LOH and no changes at high frequencies.

It is interesting that several cancer registries have shown that patients with endometriosis have an increased risk of ovarian cancer (26–29). From our study, we cannot exclude the possibility that endometriosis is a precursor to endometrioid and clear cell ovarian cancer since we may have overlooked LOH at other loci or genetic alterations other than LOH may be involved. In addition, some endometriotic lesions may gain a neoplastic component at a later stage; many of our samples were from young women (mean age, 37 years), and no atypia was identified in any of the lesions and none were associated with ovarian cancer. Similar risk factors appear to be involved in both endometriosis and ovarian cancer, and there may be an indirect link between the two diseases where environmental, immunological, hormonal, and genetic factors coincidentally predispose women to both endometriosis and ovarian cancer.

In summary, our data do not provide good evidence for the "endometriosis-as-tumor" hypothesis. We failed to replicate previous findings of LOH in ovarian endometriosis. Evidently, we cannot exclude the possibility that endometriosis is neoplastic because we may have overlooked LOH at other loci or genetic lesions other than LOH may be involved. Other genetic methods, such as linkage analysis in familial cases and profiling of gene expression, may yield more useful information about the origins of endometriosis. Finally, direct analysis of somatic genetic changes in endometriosis might be better focused on late-stage lesions or lesions associated with contiguous carcinoma.

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