

# Microsatellite DNA assays reveal an allelic imbalance in p16<sup>Ink4</sup>, GALT, p53, and APOA2 loci in patients with endometriosis

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**Objective:** To detect allelic imbalance on specific genetic loci occurring in endometriosis.

**Design:** Microsatellite analysis.

**Setting:** Paraffin-embedded tissues histologically confirmed as endometriotic or normal endometrium.

**Patient(s):** Premenopausal women undergoing laparoscopy for suspected endometriosis.

**Intervention(s):** Laparoscopic excision of specimens.

**Main Outcome Measure(s):** Allelic imbalance and alterations of intensity of microsatellite alleles.

**Result(s):** Five of 17 microsatellite DNA markers (29.4%) showed allelic imbalance. Eight samples (36.4%) showed allelic imbalance in at least one locus. Loci 9p21, 1q21, and 17p13.1 exhibited imbalance in 27.3%, 4.5%, and 4.5%, respectively. A 3-fold increase of the fractional allelic loss was observed from disease stage II to III and IV, whereas only 1.3-fold was found between patients of 41–50 and 20–40 years.

**Conclusion(s):** We found that loss of heterozygosity on p16<sup>Ink4</sup>, GALT, and p53, as well as on APOA2, a region frequently lost in ovarian cancer, occurs in endometriosis, even in stage II of the disease. The occurrence of such genomic alterations may represent important events in the development of endometriosis. The 9p21 locus may contain a gene associated with the pathogenesis of the disease, and therefore its loss may be a prognostic marker of the disease. (*Fertil Steril*® 2001;75:160–5. ©2001 by American Society for Reproductive Medicine.)

**Key Words:** Endometriosis, loss of heterozygosity, p16<sup>Ink4</sup>, GALT, p53, APOA2

Several lines of evidence provide two hypotheses in the effort to identify the pathogenesis of endometriosis. Some studies, using genetic polymorphisms and linkage analysis, implicate genes that encode for metabolic and detoxification enzymes, such as galactose 1-phosphate uridyl transferase (GALT), located on 9p21 (1, 2), and glutathione S-transferase M1 (GSTM1), located on 1p13 (3, 4), respectively, in the pathogenesis of the disease. The second hypothesis proposes endometriosis as a disorder that predisposes to cancer. Although endometriosis is considered to be a benign condition, it is sometimes found in close association with ovarian carcinoma, particularly the endometrioid and clear cell subtypes, which suggests malignant progression (5–8). Moreover, the glandular epithelium of endometriosis can show cytologic atypia (9),

and there is evidence that DNA aneuploidy and loss of heterozygosity occur (10, 11).

These studies demonstrate tumor suppressor genes as crucial factors for the development of endometriosis. The above hypotheses indicate that endometriosis is a complex trait in which multiple genetic loci, with different functions, interact with each other and the environment via different pathways to produce the disease phenotype (12).

An array of DNA-repair systems function in the cell to avoid the effects of accumulation of DNA alterations. The DNA mismatch repair system plays a crucial role in this process by counteracting effects caused by DNA damage, genetic recombination, or replication errors. In humans, two different heterodimeric complexes of MutS-related proteins (hMSH2-hMSH3 and hMSH2-hMSH6) and two differ-

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TABLE 1

Microsatellite DNA markers studied.

Locus	Cytogenetic location	Observed heterozygosity	Allele size range (bp)	Proximal genes	Primer concentration in reaction (nM)	Panel <sup>a</sup>
D1S116	1p31-p21	0.65	89-101	—	100	A
D1S186	1p	0.84	82-106	—	100	B
ACTN2	1q42-q43	0.50	105	—	100	— <sup>b</sup>
APOA2	1q21-q23	0.70	131-145	—	120	A
D2S2291	2p	0.76	245	MSH6, MSH2	140	A
D2S288	2p	0.62	276-284	MSH6, MSH2	147	B
D2S141	2q21-q33	0.88	152-178	PMS1	135	B
D2S202	2q32	0.93	135-207	PMS1	120	C
D2S311	2q	0.81	185-207	PMS1	135	C
D3S1561	3p24.2-p22	0.65	226-250	MLH1	145	D
D9S161	9p21	0.78	119-135	p16, GALT	80	A
D9S270	9p21	0.71	87-101	p16, GALT	100	C
IFNA	9p22	0.72	138-150	p16, GALT	120	B
D9S265	9p21	0.61	84-100	p16, GALT	80	D
D9S112	9q31-q34	0.88	112-135	—	120	C
TP53	17p13.1	0.90	103-135	p53	100	D
D17S250	17q11.2-12.1	0.91	151-169	—	135	D

<sup>a</sup> Panel of 4-plex reaction.<sup>b</sup> Single PCR assay.Goumenou. Microsatellite DNA assays. *Fertil Steril* 2001.

ent heterodimeric complexes of MutL-related proteins (hMLH1-hPMS2 and hMLH1-hPMS1) have been characterized as fundamental for the proper function of DNA mismatch repair in both base and insertion/deletion mispairing (13).

In this context, we have used polymerase chain reaction (PCR)-based microsatellite DNA analysis to investigate the probability of allelic imbalance (also termed loss of heterozygosity) in candidate genetic loci involved in susceptibility to endometriosis. Although the detection of an allelic imbalance alone cannot support a full gene-inactivation hypothesis, it is a direct indication of genomic instability. The frequency of its occurrence in a specific chromosomal region in concordance with a specific phenotype may have functional significance.

## MATERIALS AND METHODS

### Specimens

Endometriosis and corresponding normal endometrial tissue samples were obtained from 22 women aged 22-55 years (mean  $\pm$  SD, 37.0  $\pm$  8.9 years) undergoing laparoscopy for suspected endometriosis at the Department of Obstetrics and Gynecology of the University Hospital of Heraklion, Crete. At the time of laparoscopy, the pelvic organs were examined for the presence and the extent of endometriosis. The disease was staged according to the revised American Fertility Society classification (14). Tissue samples from endometriotic cysts and normal endometrium were collected during hysteroscopy. The specimens were obtained

from ovarian endometriotic tissue, embedded in paraffin, and studied histologically. The University of Crete ethics committee approved this study, and all patients gave written informed consent.

### DNA Extraction

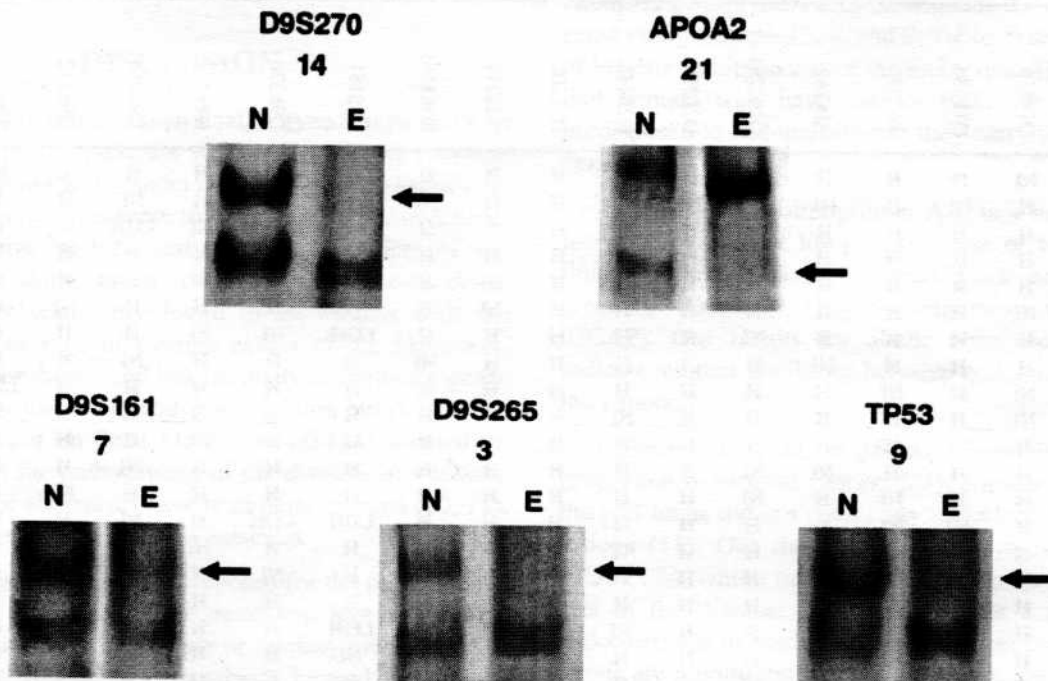
Twenty serial 15- $\mu$ m-thick sections of each paraffin-embedded sample were dewaxed and lysed in 400 mM Tris-HCl, pH 8.0, 150 mM NaCl, 60 mM EDTA, 1% sodium dodecyl sulfate, and 10  $\mu$ g/mL proteinase K and incubated at 65°C for 16 hours in an orbital shaker. The endometriotic tissue specimens were microdissected from serial 15- $\mu$ m-thick sections to yield  $\geq$ 70% of endometriotic cells before DNA preparation (15). Deproteinization included extraction with phenol/chloroform and chloroform. DNA was precipitated by the addition of an equal volume of isopropanol. DNA was washed with 70% ethanol and resuspended in 50  $\mu$ L of buffer containing 10 mM Tris-HCl and 1 mM EDTA, pH 8.0. Working stocks were prepared by 10-fold dilution in double-distilled H<sub>2</sub>O.

### Primers and PCR Amplification of Microsatellite Loci

Seventeen microsatellite DNA markers (Research Genetics, USA) were studied, located in chromosomal regions 1p31-p21, 1q21-q23, 1q42-43, 2p22-p21, 2q21-q33, 3p24.2-p22, 9p22-p21, 9q31-q34, 17p13.1, and 17q11.2-q12.1. We selected these regions because 1q21 is commonly lost in ovarian neoplasms (The Cancer Anatomy Project, CCAP, <http://www.ncbi.nlm.nih.gov/CCAP>) (16), whereas MSH2 has been mapped to 2p22-p21, MSH6 to 2p16, PMS1 to

**FIGURE 1**

Representative examples of specimens exhibiting loss of heterozygosity. N = normal endometrial DNA; E = endometriotic DNA. Arrows indicate the position of a deleted allele. The faint bands in the position of the deleted alleles are attributed to contamination with normal DNA derived primarily from peripheral blood. The numbers below the locus name represent the patient numbers.



Goumenou. Microsatellite DNA assays. *Fertil Steril* 2001.

2q24.1-q32.1, MLH1 to 3p21.32-p21.1, tumor suppressor gene p16<sup>Ink4</sup> to 9p21, GALT to the same region, and tumor suppressor gene p53 to 17p13.1 (data from Genome Database, <http://gdbwww.gdb.org>). The other markers are not related to endometriosis and were used to evaluate the integrity of the genome.

Microsatellite DNA markers were amplified in four panels of 4-plex reactions and one single assay (Table 1). We introduced 100 ng of genomic DNA in a PCR reaction mixture containing 1× PCR buffer, 350 μM dNTPs, 2.66 mM MgCl<sub>2</sub>, and 0.35 U *Taq* DNA polymerase (GIBCO BRL, Life Technologies Inc., Gaithersburg, Scotland, UK). To optimize 4-plex reactions, we used different concentrations of each marker primer set (Table 1). Amplification parameters were as follows: initial denaturation for 3 minutes; 30 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds; and a final extension step at 72°C for 10 minutes (17). PCR products were subjected to electrophoresis in a 10% polyacrylamide gel and silver stained. Loss of heterozygosity was scored when a significant reduction in the intensity of one allele in the heterozygous specimen was observed in the endometriotic DNA. In cases

positive for loss of heterozygosity, the analysis was repeated three times and the results were 95% reproducible.

## RESULTS

We assayed 22 endometriotic/normal DNA pairs with a total of 17 microsatellite markers. The incidence of imbalance for each marker ranged from 0% to 17.6%, whereas the degree of heterozygosity ranged from 0.50 to 0.93 (Table 1). Characteristic examples of allelic imbalance are shown in Figure 1. Eight specimens (36.4%) showed allelic imbalance in at least one of the examined loci. Allelic imbalance was observed in five microsatellite DNA markers in D9S270 (17.6%), D9S161 (15.0%), D9S112 (6.7%), APOA2 (5.5%), and TP53 (4.8%). Homozygote genotype of a sample in a microsatellite DNA marker was considered to be noninformative in analysis of loss of heterozygosity. An analytic presentation of the individual allelotypes for the 22 endometriotic specimens is given in Table 2.

The value for fractional allelic imbalance was calculated for each sample as: (loci scored with allelic imbalance)/(total informative loci) (17). The highest value of this factor was 0.14 in a patient with stage III endometriosis, aged 42 years.

**TABLE 2**

Individual allelotypes for the 22 endometriotic cases studied.

Patient no.	Age (y)	Stage of endometriosis	Microsatellite DNA markers																	Fractional allelic loss
			D1S116	D1S186	ACTN2	APOA2	D2S2291	D2S288	D2S141	D2S202	D2S311	D3S1561	D9S161	D9S270	IFNA	D9S265	D9S112	TP53	D17S250	
1	44	II	NI	H	H	H	H	H	H	H	H	H	H	NI	H	H	NI	NI	H	0.00
2	35	III	H	NI	H	NI	H	NI	H	H	H	NI	H	H	H	NI	H	H	H	0.00
3	35	IV	H	H	H	H	H	H	H	H	H	H	H	NI	NI	LOH	NI	H	H	0.07
4	45	III	H	H	H	H	H	H	H	H	H	H	H	NI	H	H	H	H	H	0.00
5	25	III	H	H	H	H	H	H	H	H	NI	H	H	H	H	H	H	H	H	0.00
6	25	IV	NI	H	H	H	H	H	H	H	NI	H	H	H	H	H	NI	H	H	0.00
7	31	III	H	H	H	H	NI	H	H	H	H	H	LOH	NI	NI	H	H	H	H	0.07
8	22	IV	H	H	H	NI	H	H	H	H	H	NI	H	H	H	NI	H	H	H	0.00
9	38	IV	NI	H	NI	H	H	H	H	H	H	H	H	H	H	H	H	LOH	H	0.07
10	31	III	NI	H	H	H	H	H	NI	H	H	H	H	NI	H	H	H	H	NI	0.00
11	40	III	H	NI	H	H	H	H	H	H	NI	H	H	H	NI	H	H	H	H	0.00
12	45	II	H	H	H	NI	NI	H	H	H	H	H	H	H	H	NI	H	H	H	0.00
13	32	IV	H	H	NI	H	NI	H	H	H	H	H	H	H	H	H	NI	H	H	0.00
14	42	III	H	NI	NI	H	H	H	H	H	H	H	LOH	LOH	H	NI	H	H	H	0.14
15	55	III	H	H	NI	H	H	H	H	H	NI	H	H	H	NI	NI	H	H	H	0.00
16	34	IV	H	H	NI	H	H	H	H	H	H	H	H	NI	H	H	H	H	H	0.00
17	47	II	H	H	H	H	H	H	H	H	H	H	H	H	H	NI	H	H	H	0.00
18	45	III	H	H	H	NI	H	H	NI	H	H	H	LOH	H	H	H	H	H	H	0.07
19	42	II	H	H	H	H	H	H	H	H	H	H	NI	H	H	H	H	H	H	0.00
20	48	II	H	H	H	LOH	NI	NI	H	H	H	H	H	H	H	H	H	H	H	0.07
21	29	IV	H	H	NI	H	H	H	H	H	H	H	NI	LOH	H	NI	NI	H	H	0.08
22	25	III	NI	H	NI	H	H	H	H	H	H	H	H	LOH	H	H	NI	H	H	0.07

Allelic imbalance/

Total informative loci (%)	0/17	0/19	0/15	1/18 (5.5)	0/18	0/20	0/20	0/22	0/18	0/20	3/20 (15.0)	3/17 (17.6)	0/15	1/15 (6.7)	0/15	1/21 (4.8)	0/21
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Note: NI = noninformative; H = heterozygosity; LOH = loss of heterozygosity.

Goumenou. Microsatellite DNA assays. Fertil Steril 2001.

A 3-fold difference was observed in the fractional allelic imbalance value between stage II and III or IV of endometriosis (Table 3). Moreover, only a 1.3-fold difference was observed in correlation with patient's age (Table 4). The fractional regional loss values were calculated for each sam-

ple as: (loci on the arm with allelic imbalance)/(total informative loci on the arm) (17). These results were 0.135, 0.056, and 0.048 for 9p21, 1q21-q23, and the 17p13.1 locus, respectively. Six of 22 samples (27.3%) showed allelic im-

**TABLE 3**

Allelic imbalance by stage of endometriosis.

Stage of endometriosis	No. of cases	Years of age <sup>a</sup>	Fractional allelic loss <sup>a</sup>
II	5	45.2 ± 2.4	0.01 ± 0.03
III	10	37.4 ± 9.7	0.03 ± 0.05
IV	7	30.7 ± 5.7	0.03 ± 0.04

<sup>a</sup> Mean ± SD.

Goumenou. Microsatellite DNA assays. Fertil Steril 2001.

**TABLE 4**

Allelic imbalance analyzed by age.

Years of age cluster	No. of cases	Fractional allelic loss <sup>a</sup>
20-30	5	0.03 ± 0.04
31-40	8	0.03 ± 0.04
41-50	8	0.04 ± 0.05
>50	1	0

<sup>a</sup> Mean ± SD.

Goumenou. Microsatellite DNA assays. Fertil Steril 2001.

balance on 9p21, compared with only one on locus 1q21-q23 (4.5%) and one on 17p13.1 (4.5%).

Microsatellite alterations were not observed in any locus because no novel generated microsatellite allele was observed in endometriotic DNA compared with the normal sample examined.

## DISCUSSION

Endometriosis is a common benign gynecologic disorder characterized by the presence and proliferation of endometrial tissue (stroma and glands) outside the endometrial cavity. Endometriotic cysts seem to be monoclonal and demonstrate aggressive growth and localized invasion of the myometrium. Malignant transformation has been documented and is commonly found in association with the endometrioid subtype of ovarian cancer (5–8). Moreover, genetic polymorphisms and linkage analysis implicate genes that encode for metabolic and detoxification enzymes, such as GALT located on 9p21 (1, 2) and GSTM1 located on 1p13 (3, 4), in the pathogenesis of the disease. In addition, because genetic alterations have been demonstrated (10, 11), DNA repair gene failure can be assumed.

Three pathways have been proposed for the full inactivation of a gene allele: intragenic mutations, loss of chromosomal material (allelic imbalance or homozygous deletion), and DNA methylation of CpG islands located in the promoter of the gene (18).

A previous report of allelic imbalance in endometriosis reported loss of heterozygosity in 28% of cases of microdissected endometriosis, most frequently on 9p (18%) and 11q (18%), but not on 17p (11). By fluorescence in situ hybridization, the number of chromosome-17 aneuploid endometriotic cells was found to be between 38% and 80%, with a mean of 65%, compared with a mean of 25% in normal endometrial cells (10).

Our microsatellite DNA assays for the 17 examined markers demonstrated the incidence of allelic imbalance to be 36.4%. The 9p21 locus showed loss in 27.3% of cases, 6-fold more frequently than 1q21-q23 or 17p13.1. These findings suggest the involvement of these loci in endometriosis. Moreover, we observed these alterations at similar rates among different patient age groups, but the rate increased 3-fold from stage II to III and IV of endometriosis, suggesting that allelic imbalance is independent of patient's age and accumulates during development of the disease.

The similarities between endometriosis and cancer (5–8) lead to the assumption that 9p21 allelic imbalance, as observed in this study, could be implicated in endometriosis by loss of a p16<sup>Ink4</sup> functional allele. On the other hand, the GALT gene, which is located on 9p13 (19), is implicated in female infertility because of ovarian failure from galactosemia (20). Although the GALT gene has been associated

with endometriosis (1, 2), it is more likely that the relative polymorphism N314D (an A2744 to G2744 transition in exon 10 substituting aspartate 314 for asparagine 314) to the disease is in linkage disequilibrium with a disease susceptibility locus (12). Two patients aged 20–30 years who presented with allelic imbalance showed loss of only this locus (samples 21 and 22; Table 2), whereas the other three of the same group (samples 5, 6, and 8; Table 2) did not exhibit loss of heterozygosity in any of the loci tested. Thus, it is possible that women who have loss of 9p21 may be genetically predisposed to endometriosis if they carry the mutant GALT gene.

APOA2, like apolipoprotein A-I, is a major apolipoprotein in high-density lipoprotein. Loss of heterozygosity in this locus is common in ovarian adenocarcinoma (Cancer Genome Anatomy Project, <http://www.ncbi.nlm.nih.gov/CCAP>). Our results for allelic imbalance on this locus indicate another similarity between endometriosis and ovarian cancer.

Although mutations in p53 have been detected in a high proportion of ovarian cancers (21), a mutational analysis on the p53 locus did not detect any mutations in endometriotic lesions (11). Our data revealed loss of heterozygosity in TP53 (4%), which may implicate the gene in the pathogenesis of the disease. This demonstrates a possible role of endometriosis in predisposition to cancer because p53 alterations are a common feature of carcinogenesis (22).

In addition, we examined the incidence of allelic imbalance in genes of the DNA mismatch repair system (MSH2, MSH6, MLH1, and PMS1). These genes play a key role in the preservation of genomic stability, which is well established from both biochemical (13) and genetic alteration studies (23) from bacteria to humans. Although genomic alterations occur in endometriosis according to previous studies (10, 11) as well as the present report, MSH2, MSH6, MLH1, and PMS1 remain unaffected by allelic imbalance. This finding implies that the DNA mismatch repair system is not responsible for the increased genomic alterations in endometriosis.

This study has produced new information on the candidate susceptibility loci involved in endometriosis. We showed the incidence of allelic imbalance in the p16<sup>Ink4</sup>-GALT region, APOA2, and on p53 loci with different subcellular functions. Allelic imbalance was seen even in stage II of the disease, independent of the patient's age, and increased dramatically from stage II to III, which implies an unknown susceptibility factor for endometriosis, possibly located on the 9p21 genetic locus. This locus may provide a prognostic marker for the early diagnosis of endometriosis.

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