Loss of heterozygosity in adenomyosis on *hMSH2*, *hMLH1*, *p16^{Ink4}* and *GALT* loci

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Abstract. Adenomyosis is a gynecological condition in which tissue histologically similar to that in endometrium is found within the myometrium in the uterus. Although, lesions of both adenomyosis and endometriosis are identical to their sources with respect to structure and function, they are generally regarded as separate and distinct nosologic processes. In this study, we used 17 microsatellite markers, in four tetraplex and one single PCR assay, to determine the incidence of loss of heterozygosity (LOH) in 31 cases of adenomyosis. The markers used are located close to tumor suppressor genes, DNA repair genes, and genes which are thought to be involved in endometriosis. Moreover, the markers were involved in regions frequently lost in ovarian cancer, on chromosomal arms 1p, 1q, 2p, 2q, 3p, 9p, 9q, 17p and 17q. Nine samples (29.0%) showed LOH in at least one locus. Loci 2p22.3-p16.1, 3p24.2-p22 and 9p21 exhibited imbalance (19.4%, 9.7% and 6.5% respectively). This is the first report, that LOH occurs in adenomyosis. The regional chromosomal losses were detectable early during the development of this condition. In addition, *hMSH2*, *hMLH1*, *p16^{Ink4}* and *GALT* genes were associated for the first time with adenomyosis and its pathogenesis.

Introduction

Adenomyosis is characterized by an abnormal growth of endometrial glands and stroma into and beyond the myometrial layers, in women of various ages. It is commonly associated with dysmenorrhea, hypermenorrhea and infertility and occurs spontaneously with age in experimental animals. Although, lesions of both adenomyosis and endometriosis are identical to their sources with respect to structure and function, as well as response to cyclic variations of endogenous ovarian steroids, they are generally regarded as separate and distinct nosologic processes (1). The etiology of adenomyosis is not known, but several pathophysiologic mechanisms have been suggested.

There are only a few reports on adenomyosis. However, several hypotheses have been proposed in the effort to identify the pathogenesis of the disease. The development of adenomyosis in animals with advanced maternal age has been reported in various species, including non-human primates (2,3). An elevated local prolactin level, in a mouse model, seems to be the endocrine trigger, with estrogen and progesterone having roles as growth promoters (4). In human, the glands of adenomyosis may selectively express more human chorionic gonadotropin/luteinizing hormone (hCG/LH) receptor mRNA and immunoreactive receptor protein than normal endometrial glands in the same specimen (5). Reactive oxygen species may also play a role in the pathogenesis of adenomyosis. The inflammation of adenomyosis produces oxygen free radicals that may induce superoxide dismutase in ectopic endometrium (6). A possible hereditary factor in the occurrence of adenomyosis have been suggested, either autosomal or X-linked dominant inherited (7,8).

This condition is closely associated with parity, because adenomyosis in human is a frequent histologic finding in uterine specimens where hysterectomy is performed for menorrhagia and dysmenorrhea (9,10).

An array of DNA repair systems function in the cell, in order to avoid the effects of accumulation of DNA alterations. One of them, the DNA mismatch repair system (MMR), plays a crucial role in this process by counteracting effects caused by DNA damage, genetic recombination or replication errors. In human, two different heterodimeric complexes of MutS-related proteins (hMSH2-hMSH3 and hMSH2-hMSH6) and two different heterodimeric complexes of MutL-related proteins (hMLH1-hPMS2 and hMLH1-hPMS1) have been characterized as fundamentals for the proper function of MMR, in both base and insertion/deletion mispairing (11,12).

Loss of heterozygosity (LOH) using highly polymorphic microsatellite markers is the most common methodology employed for the localization of sites in the genome with high probability for the presence of candidate dominant genes (13). The finding of LOH at a microsatellite marker locus implies that a nearby gene could be affected by the genetic lesion (14). The highly polymorphic microsatellite DNA, composed of repetitive sequences of dinucleotides (CA)n and rarely trinucleotides, has been used for genetic linkage analysis, in various neoplastic and non-neoplastic conditions (15).

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Table I. Microsatellite DNA markers studied.

Locus	Cytogenetic location	Het[a]	ASR[b] (bp)	Proximal genes	Primer concentration in reaction (nM)	Panel[c]
D1S116	1p31-p21	0.65	89-101		100	A
D1S186	1p	0.84	82-106		100	B
ACTN2	1q42-q43	0.50	105		100	-[d]
APOA2	1q21-q23	0.70	131-145		120	A
D2S2291	2p22.3-p16.1	0.76	245	*hMSH2*	140	A
D2S288	2p22.3-p16.1	0.62	276-284	*hMSH2*	147	B
D2S141	2q21-q33	0.88	152-178	*hPMS1*	135	B
D2S202	2q32	0.93	135-207	*hPMS1*	120	C
D2S311	2q	0.81	185-207	*hPMS1*	135	C
D3S1561	3p24.2-p22	0.65	226-250	*hMLH1*	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

[a]Het, observed heterozygosity. [b]ASR, allele size range. [c]Panel of 4-plex reaction. [d]Single PCR assay.

The aim of this study was to evaluate the existence of LOH in adenomyotic tissues possibly implicated in the pathogenesis of the disease, as well as the correlation of this genetic alteration with clinical and epidemiological parameters.

Materials and methods

Specimens. Adenomyosis and corresponding normal endometrial tissue samples were obtained from 31 women aged 33-72 (52±11) years undergoing hysterectomy for menorrhagia and dysmenorrhea at the Department of Obstetrics and Gynecology of the University Hospital of Heraklion, Crete, from January 1993 to January 1998. Tissue samples were obtained during operations. Adenomyosis was diagnosed by the presence of normal appearing endometrial glands and stroma, one or more standards low-power fields away from the endometrial myometrial junction (16). The menstrual cycle was divided into three phases according to the histopathologic evaluation: proliferative (n=13), secretory (n=8) and post-menopausal (n=10). The University of Crete ethics committee approved this study and all the patients gave written informed consent.

DNA extraction. Twenty serial, 15-μm 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% SDS, 100 μg/ml Proteinase K and incubated at 65°C for 16 h in an orbital shaker. 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 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Working stocks were prepared by 10-fold dilution in double distilled H_2O.

Primers and multiplex 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 usually lost in ovarian neoplasms (The Cancer Anatomy Project, CGAP, http://www.ncbi.nlm.nih.gov/CCAP) (17), *hMSH2* has been mapped to 2p22-p21, *hPMS1* to 2q24.1-q32.1, *hMLH1* to 3p21.32-p21.1, tumor suppressor gene $p16^{Ink4}$ to 9p21, *GALT* to the same region, tumor suppressor gene *p53* to 17p13.1 (data from GenomeDatabase, http://gdbwww.gdb.org) and the other markers as not related to endometriosis, in order to evaluate the integrity of the genome. Microsatellite DNA markers were amplified in four panels of 4-plex reactions and one single assay (Table I). We introduced 100 ng of genomic DNA in a PCR reaction mixture containing 1X PCR buffer, 350 μM dNTPs, 2.66 mM $MgCl_2$ and 0.35U Taq DNA polymerase (Gibco BRL, Life Technologies). To optimize 4-plex reactions, different concentrations of each marker primer set were used (Table I). Amplification parameters were the following: initial denaturation for 3 min; 30 cycles consisted of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec; final extension step at 72°C for 10 min (18). PCR products were

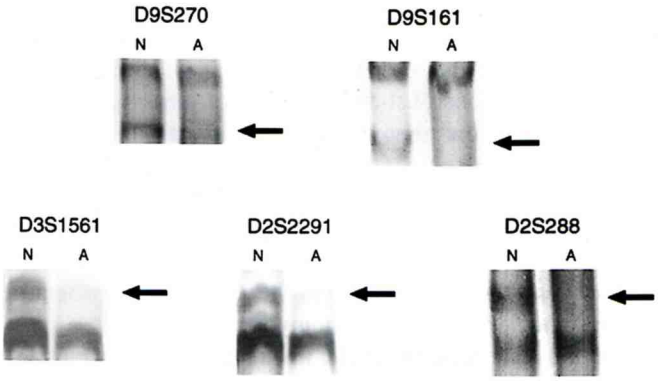

Figure 1. Representative examples of specimens exhibiting LOH. A, adenomyotic DNA. N, normal endometrial DNA. Arrows indicate the position of a deleted allele. The faint bands in the position of the deleted alleles are attributed to the presence of normal DNA derived from peripheral blood. The numbers above the locus name represent the patient numbers.

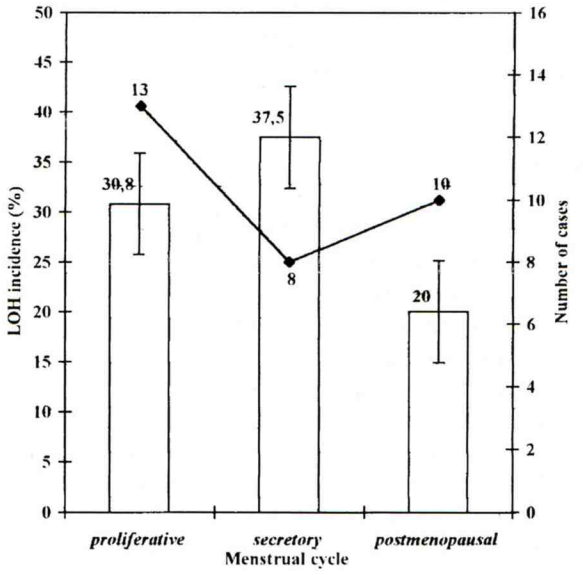

Figure 2. LOH incidence values in adenomyosis in association with the menstrual cycle. The vertical bars represent the LOH incidence, while the line represents the number of the cases in each menstrual cycle. The Y-error bars provide the standard error.

electrophoresed in a 10% polyacrylamide gel and silver stained. LOH was scored when a significant reduction (>90%) in the intensity of one allele in the heterozygous specimens was observed compared with the other allele in the adenomyotic DNA. The analysis in the LOH positive cases was repeated three times and the results were reproducible.

Statistical analysis. Single factor analysis of variance (ANOVA) and unpaired Student's t-test to examine the data versus the clinicopathological parameters were used (19). Our 99% reference range was constructed conservatively as mean ± 3SD.

Results

We assayed 31 adenomyotic/normal DNA pairs with a total of 17 microsatellite markers. The incidence of imbalance for each marker ranged from 0% to 12.9%, while the degree of heterozygosity was from 0.50 to 0.93 (Table I). Characteristic examples of allelic imbalance are shown in Fig. 1. Nine specimens (29.0%) showed LOH in at least one of the examined loci. Allelic imbalance was most frequently observed in D2S2291 (12.9%) followed by D3S1561 (9.7%). Homozygote genotype of a sample in a microsatellite DNA marker was considered to be non-informative in LOH analysis. Microsatellite alterations were not observed in any loci, since no novel generated microsatellite allele was observed in adenomyotic DNA, compared to the normal sample examined.

The fractional allelic imbalance value was calculated for each sample as [loci scored with allelic imbalance (LOH)]/(total informative loci) (18). The highest value of the factor was 0.125 in a proliferative case, aged 38-years.

The LOH incidence was examined according to clinicopathological parameters such as menstrual cycle and patient's age. Fig. 2 provides the LOH incidence values in women with adenomyosis in association with menstrual cycle. A slight decline in the percentage of LOH incidence from secretory to proliferative and to postmenopausal phase is apparent. However, this decline was not statistically significant.

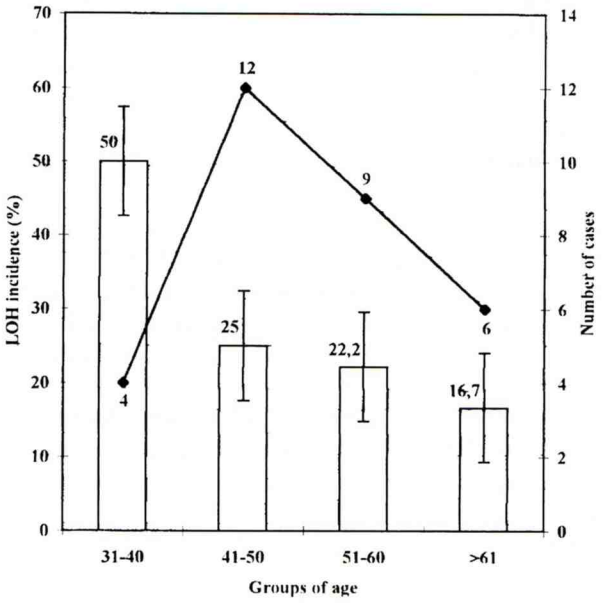

Figure 3. The LOH incidence values in women with adenomyosis in association with patient's age. Patients were divided into four age groups. The vertical bars represent the LOH incidence, while the line represents the number of the cases in each age group. The Y-error bars provide the standard error.

Patients were divided into 4 age groups (31-40, 41-50, 51-60 and >61 years of age) and LOH incidence was calculated for each group (Fig. 3). Although, the number of cases in the 31-40 years age group was relatively small (only 4 cases) and inappropriate for statistical evaluation, it is noteworthy that two patients out of four in this group, exhibited LOH, one in D2S2291 locus and the other in both D3S1561 and D9S161 loci.

The regional allelic loss of heterozygosity was 19.4%, 9.7% and 6.5%, on 2p22.3-p16.1, 3p24.2-p22 and 9p21 chromosomal regions, where *hMSH2*, *hMLH1*, *p16^{Ink4}* and *GALT* genes are located respectively.

Discussion

Adenomyosis is an enigmatic and elusive gynecological condition contributing to menorrhagia, progressive dysmenorrhea and diffuse enlargement of the uterus in women of late and post reproductive age. Ectopic but intramyometrial, uterine glands and stroma constitute a paraphysiologic state in some affected women or a pathologic condition more closely related to endometriosis. It remains among the least-studied gynecologic disorders, although it is diagnosed in one in three hysterectomy specimens. Furthermore, adenomyosis can also lead to infertility in women of middle and late reproductive age, causing early miscarriage of the embryo, because of abnormal immune responses of the affected endometrial environment (20).

Endometriosis and adenomyosis, have both associations with autoantibody formation (20,21) and apoptosis (22,23). Moreover, genetic polymorphisms and linkage analysis, implicate genes which encode for metabolic and detoxification enzymes, such as galactose 1-phosphate uridyl tranferase (*GALT*) located on 9p21 (24,25) and glutathione S-transferase M1 (GSTM1) located on 1p13 (26,27), in the pathogenesis of endometriosis. In addition, microsatellite analysis of endometriosis has revealed LOH at candidate ovarian tumor suppressor gene loci (28).

We used PCR-based microsatellite DNA analysis to investigate the probability of LOH in candidate endometriosis gene loci, and mismatch repair (MMR) genes in adenomyotic tissue. Genomic instability, in the form of LOH, is probably the most useful marker to identify the presence of candidate dominant genes associated with a particular disease phenotype. Multiplex PCR-based assays can facilitate this due to their ability to amplify DNA from minute amounts of tissue, with several different microsatellite DNA markers. Moreover, this makes it ideal for screening large numbers of DNA from clinical samples, in the early detection of genetically abnormal cells and thus assist early diagnosis.

Our set of data of multiplex PCR-based assays, for the 17 examined microsatellite DNA markers demonstrated the incidence of LOH on 2p22.3-p16.1, 3p24.2-p22 and 9p21 chromosomal regions, as 19.4%, 9.7% and 6.5% respectively. These findings suggest the implication of these genetic loci in adenomyosis. Moreover, the above genomic alterations were observed in two out of four cases of women in middle reproductive age, thus it is possible that these aberrations are involved in the initiation of adenomyosis and the early onset of the disorder.

The MMR system, which is involved in increasing the fidelity of replication by specific repair of DNA polymerase incorporation errors, was discovered earlier in prokaryotes, but has been shown to be involved in cancer only within the past five years. Mutations in mismatch repair genes leads to cancer predisposition (29). The complete loss of function of MMR genes creates a characteristic mutator phenotype known as microsatellite instability (30). Our findings for LOH on 2p22.3-p16.1 and 3p24.2-p22, where *hMSH2* and *hMLH1* are located, leads to the assumption that in certain cases of adenomyosis, cancer predisposition could be suggested. Because of the importance of these genes for the preservation of the genome from mutations, an error prone phenotype could be assumed. Moreover, in a set of 23 endometriotic tissues and corresponding normal endometrium microsatellite DNA PCR-based assays, carried out in our laboratory with the same genetic markers, these genetic loci remain unaffected (31). This is the first report associating *hMSH2* and *hMLH1* with adenomyosis.

On the other hand the allelic imbalance on 9p21, could lead to two assumptions. First, to the implication of *p16^{Ink4}*, a tumor suppressor gene locus with adenomyosis, which contributes to further similarities of adenomyosis to cancer. Second, to the implication of the *GALT* gene, also located on 9p21, in this gynecologic disorder. *GALT* gene has been associated with female infertility because of ovarian failure from galactosemia (32) and with endometriosis. LOH of this locus was reported in endometriosis (28), which implies a similarity of adenomyosis to this disease.

This study has produced new information on the candidate susceptibility loci, involved in adenomyosis. We showed, for the first time, the incidence of LOH on *hMSH2*, *hMLH1* and *p16^{Ink4}-GALT* genetic regions, loci with different subcellular functions. Moreover, we introduced a new genetic approach to this disorder, with possible usage for diagnostic purposes. The pathogenesis of adenomyosis has unique features distinct from endometriosis and certain cases should be examined as a precancerous situation.

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