Microsatellite Analysis of Endometriosis Reveals Loss of Heterozygosity at Candidate Ovarian Tumor Suppressor Gene Loci

Xiu Xian Jiang, Andrew Hitchcock, Emma J. Bryan, Richard H. Watson, Patricia Englefield, Eric J. Thomas, and Ian G. Campbell

Obstetrics and Gynecology, University of Southampton, Princess Anne Hospital, Cowford Road, Southampton SO16 5YA [X. J., E. J. B., R. H. W., P. E., E. J. T., I. G. C.], and Department of Histopathology, Southampton General Hospital, Tremona Road, Southampton SO16 6YD [A. H.], United Kingdom

ABSTRACT

Endometriosis is a very common gynecological condition in which tissue similar to endometrium proliferates at sites outside the uterine cavity, most commonly the ovary. Although it generally remains a benign condition, malignant transformation has been documented, and it is commonly found in association with endometrioid subtype ovarian cancer. Tumor suppressor genes are commonly altered in ovarian cancers, and the development of endometriosis may involve mutations in the same class of genes. We have investigated this possibility by examining DNA from 40 cases of endometriosis for clonal status, alterations in TP53 and RASK, and allelic losses at candidate ovarian tumor suppressor loci on chromosome arms 6q, 9p, 11q, 17p, 17q, and 22q. The majority of endometriotic cysts were monoclonal, but interestingly, 8 of 10 normal endometrial glands were also monoclonal, demonstrating that both can develop from a single progenitor cell. No mutations were detected in TP53 or RASK, and loss of heterozygosity (LOH) was detected on chromosomes 9p (18%), 11q (18%), and 22q (15%). In total, 11 of 40 (28%) cases demonstrated LOH at one or more of these loci. This study, which is the first to report LOH in endometriosis, supports the notion that tumor suppressor gene inactivation may play a role in the development of at least a subset of cases.

INTRODUCTION

Endometriosis is a condition in which tissue histologically similar to endometrium is found at sites outside the uterine cavity. The pathogenesis of the disease is uncertain, but implantation of viable endometrium refluxed into the peritoneal cavity during menstruation and metaplasia of peritoneal epithelium are two major theories invoked to explain its origin (1). Endometriosis is one of the most commonly encountered diseases in gynecology, requiring medical and often surgical treatment. The reported incidence of the disease has increased sharply over recent years (2), and although the exact incidence is difficult to assess, it has been recorded in up to 25% of all gynecological laparotomies and laparoscopies (3, 4). A respectable body of opinion now considers that the appearance of a small amount of endometriosis may be a physiological phenomenon in all women at some time in their reproductive career, only becoming a progressive and destructive disorder in a few.

Although endometriosis is generally considered to be a benign condition, several lines of evidence support the hypothesis that progression to frank malignancy can occur: (a) endometriosis is sometimes found in close association with ovarian carcinoma, particularly endometrioid and clear cell subtypes, suggesting malignant progression (5–8). This association is supported by Vercellini et al. (9), who, in a study of 556 patients undergoing surgery for ovarian cancers, found endometriosis in 26.3% of women with endometrioid subtype versus 3.6–5.6% with serous and mucinous subtypes; (b) the glandular epithelium of endometriosis can show cytological atypia, which is frequently associated with DNA aneuploidy (10), a phenomenon commonly seen in malignant lesions of other organs (11, 12); and (c) first degree relatives of an affected patient have an increased risk of developing the disease (13, 14) and often present with more severe pathology, suggesting an inherited genetic predisposition. These features are strikingly similar to those observed for a variety of cancers. In malignancies, advancing stages of disease are marked by the accumulation of genetic alterations in TSGs and oncogenes (15), and it is possible that genetic mutations might also be involved in the development and progression of endometriosis.

Molecular genetic data for endometriosis are scant, and as yet no specific gene mutations or other genetic lesions such as LOH, indicative of loss of TSG function, have been reported. The only study that searched for specific gene mutations in TP53 and RASK was based on only 10 samples (16). In another study, 45 endometriotic implants were examined for cytogenetic abnormalities, but no consistent alterations were observed (17). However, this might be due to limitations in the sensitivity of conventional cytogenetic techniques to subtle genetic lesions or to the overgrowth of normal cells in the short-term cultures used to analyze the karyotype.

In this study, we show that genetic deletions similar to those found in carcinomas are detectable in endometriosis, suggesting that TSG inactivation may be involved in the pathogenesis of the disease.

MATERIALS AND METHODS

Frozen Specimens. Fresh tissue specimens were obtained from 33 women undergoing surgery (laparotomy or laparoscopy) for suspected endometriosis at the Princess Anne Hospital (Southampton, United Kingdom). The tissue was snap-frozen and was stored in liquid nitrogen until use. Serial frozen sections (7-µm thick) were stained with H&E, and after examination by a gynecological pathologist (A. H.), the diagnosis of endometriosis was confirmed in 14 of the 33 cases. Selected areas showing both endometriotic glands and stroma were then dissected directly from the slide as described below. Matching normal tissue for preparation of normal DNA was obtained from a blood sample.

Archival Paraffin-embedded Specimens. Twenty-six cases of endometriosis were accessed from paraffin blocks. Serial 15-µm-thick sections were dewaxed in xylene and were rehydrated in 95% ethanol before staining with H&E. Endometriosis was reconfirmed histologically in all 26 cases.

Ovarian Cancer Specimens. Twenty-one endometrioid ovarian carcinoma samples were obtained from patients undergoing primary surgery in hospitals in and around Southampton. Blood was taken from the same individuals for preparation of normal DNA.

Microdissection and DNA Extraction. Endometriotic glands and stroma were microdissected directly from slides, based on the technique described by Pan et al. (18). One stained section was mounted with a coverslip, with the remaining adjacent serial sections being left without a coverslip for tissue removal. Using the covered H&E-stained slide as the template, areas of endometriosis that included both gland and stromal cells were scraped off using a 21-gauge needle under a dissecting microscope. Transfer of the cells was facilitated by moistening the needle with lysis buffer. Cells were recovered from a minimum of four consecutive sections. The dissected cells were frequently associated with DNA aneuploidy (10), a phenomenon commonly seen in malignant lesions of other organs (11, 12); and (c) first degree relatives of an affected patient have an increased risk of developing the disease (13, 14) and often present with more severe pathology, suggesting an inherited genetic predisposition. These features are strikingly similar to those observed for a variety of cancers. In malignancies, advancing stages of disease are marked by the accumulation of genetic alterations in TSGs and oncogenes (15), and it is possible that genetic mutations might also be involved in the development and progression of endometriosis.

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The abbreviations used are: TSG, tumor suppressor gene; LOH, loss of heterozygosity; SSCP, single-strand conformational polymorphism.
transferred to a 0.5-ml Eppendorf tube and were incubated in 100 µl of lysis buffer (50 mM Tris, 1 mM EDTA, and 0.5% Tween 20) and proteinase K (200 µg/ml) for 12–24 h at 55°C. The samples were then heated to 95°C for 10 min to destroy proteinase K activity and were stored at 4°C. Matching normal DNA for archival specimens was similarly prepared from uninvolved areas of the sections.

PCR Reactions. PCR reactions were performed in a total volume of 10 µl containing 1–3 µl of DNA, dGTP, dATP, and dCTP at a concentration of 200 µM, dCTP at a concentration of 50 µM, 0.05 µCi [α-32P]dCTP, 25 ng of each primer, and 0.5 units of Taq DNA polymerase (Promega). The PCR conditions consisted of 5 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at the appropriate annealing temperature, and 45 s at 72°C, followed by 1 cycle of 5 min at 72°C.

Clonality Analysis. Clonality analysis was performed using primers flanking a highly polymorphic trinucleotide repeat in the first exon of the human androgen receptor gene at X cen-q13 (Table 1). Methylation of the HpaII and HhaI restriction endonuclease sites in the first exon of the androgen receptor gene correlates with chromosome X inactivation (19). To test for methylation of these sites, three reactions were prepared for each specimen. Each reaction was carried out in a total volume of 20 µl and contained 10 µl of DNA in 1 × reaction buffer. No restriction enzyme was added to one tube, which acted as the control. To the second and third reaction was added 1 µl HpaII (Promega) or 1 µl HhaI, respectively. After incubation overnight at 37°C, the digestion was terminated by incubating for 10 min at 95°C. One µl of each reaction was then used for PCR amplification of the androgen receptor gene. The sequences of the primers and PCR conditions have been reported previously (19). Three µl of loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, and 30% glycerol) were added to the completed PCR reactions, and 2 µl were loaded onto 6% (29:1, acrylamide:bis-acrylamide) nondenaturing polyacrylamide gels, which were run at 200–300 V overnight at room temperature. Gels were dried and exposed to GRI X-ray film (Genetic Research Instrumentation, U.K.).

SSCP Analysis. Exons 5–8 of the TP53 gene and the RASK gene were amplified using published protocols (20). Samples were prepared and analyzed by SSCP as described previously (21).

LOH Analysis with Microsatellite Markers. The microsatellite markers used in this study and their chromosomal locations are listed in Table 1. One µl of DNA (3 µl for endometriosis case 7 and case 23) was used for PCR amplification. The PCR products were resolved on 8% nondenaturing polyacrylamide gels and were run at 200–300 V overnight at room temperature. Gels were dried and then were exposed to GRI X-ray film.

RESULTS

The detection of genetic alteration by LOH or SSCP analysis is highly dependent on the removal of the majority of normal tissue from the specimens before DNA extraction. This was especially important in this study because endometriotic implants generally represent a very low percentage of the total biopsy. To overcome this problem, we dissected endometriotic glands and adjacent endometriotic stroma directly from slides with the aid of a fine-gauge needle, using a modification of the technique described by Pan et al. (18). Fig. 1 illustrates the accuracy with which endometriotic glands and stroma were dissected from surrounding normal stroma. As a result, we estimate our samples contain >75% endometriotic tissue. To verify

![Fig. 1. H&E-stained frozen section of endometriosis (case E7) showing glandular (G) and stromal (S) cells before (A) and after (B) dissection.](image-url)
and was analyzed as described in Fig. 3A. A monoclonal pattern is seen in Lanes I and 2, and a polyclonal pattern is seen in Lane 3.

compared to the undigested DNA, was detected in endometriosis sample E12. A clonal phenotype was detected with both Hpall and HhaI in EC36, EI I, and PBS. In EC128, a clonal phenotype was detected with the Hpall digest but not with the Hhal digest. B, clonality analysis of normal endometrial glands. DNA was prepared from individual endometrial glands obtained, the technique was applied to an endometrioid ovarian cancer

The products were separated on an 8% nondenaturing polyacrylamide gel. A polyclonal phenotype, indicated by no change in the relative intensity of the band from the digested DNA and the effectiveness of the technique and to assess the quality of the DNA obtained, the technique was applied to an endometrioid ovarian cancer sample that was known to contain a TP53 mutation in exon 8. SSCP analysis of DNA from the undissected tumor biopsy shows a clear band shift compared with the matched normal DNA (Fig. 2). The DNA extracted from the dissected sample also shows a clear band shift and has significantly less normal contamination than DNA extracted from the whole biopsy.

Clonality Analysis. We examined 20 endometriosis samples and 15 normal endometrial glands for their clonal status, using a polymorphic microsatellite marker located within the androgen receptor gene. Seventeen of the endometriosis samples and 10 of the normal endometrial glands were informative at this locus and were analyzed as follows. PCR amplification was repeated, but with prior restriction endonuclease digestion with either HpaII or HhaI (Fig. 3A and 3B). Fourteen of the endometriosis samples (82%) exhibited a monoclonal pattern with either HpaII or HhaI or both (5 cases displayed monoclonal pattern with HpaII, 4 cases displayed monoclonal pattern with HhaI, and 5 cases displayed monoclonal pattern with both HpaII and HhaI). Three samples showed no preferential loss of either allele with either restriction enzyme, consistent with a polyclonal origin. These data are consistent with the smaller study of Nilbert et al. (22) and clearly demonstrate that the majority of endometriotic implants are monoclonal. Interestingly, we also observed a monoclonal pattern in 8 of the 10 (80%) normal endometrial glands, examples of which are shown in Fig. 3B. Thus, both normal and endometriotic glands seem to arise by clonal expansion from a single progenitor cell.

SSCP Analysis of TP53 and RASK. Exons 5-8 of the TP53 gene and the RASK gene were examined for mutations in all 40 endometriosis samples, but no mutations were observed in either gene (Fig. 4). Because of the association of endometriosis with endometrioid ovarian cancer and the lack of specific data in the literature concerning this particular histological subtype, 21 endometrioid carcinomas were also examined. No mutations were found in RASK, but four tumors (19%) showed tumor-specific SSCP band shifts in TP53. This frequency is considerably lower than that reported for ovarian carcinomas in general and may indicate a different developmental pathway for endometrioid carcinomas.

LOH Analysis of Candidate Tumor Suppressor Loci. Fifteen microsatellite markers were used to examine for LOH on chromosome arms 6q, 9p, 11q, 17p, 17q, and 22q. These regions were selected for the initial study because they are thought to harbor TSGs involved in the development of ovarian and other epithelial cancers (23, 24). The frequency of LOH on each of these chromosomes is shown in Table 2 together with the LOH observed in 21 endometrioid ovarian cancers. Eleven of the 40 (27.5%) endometriosis cases demonstrated LOH at one or more markers on chromosome arms 9p, 11q, and 22q as summarized in Table 3. Three cases showed LOH on chromosome 9p only, two on 11q only, and one on 22q only. The remaining five cases showed LOH on two or more of these chromosomes. Representative autoradiographs are shown in Fig. 5. In endometriosis samples E7 and E23, LOH is clearly visible, with all the informative markers on chromosomes 22q and 9p, whereas heterozygosity is clearly retained on chromosome 6q and at the TP53 locus on 17p13.1. Although it is possible that preferential amplification of one allele might account for this, we consider this unlikely as LOH was consistently observed in independent PCR reactions and with markers from different regions on chromosomes 22q and 9p. Furthermore, DNA prepared from a second set of slides yielded the same LOH patterns.

In the endometrioid ovarian carcinomas, the frequency of LOH on chromosomes 6q, 9p, 11q, and 17q (Table 2) was in keeping with previous studies in ovarian cancers (23, 24). In contrast, LOH on...
Table 2 LOH and TP53 mutation data for endometriosis and endometrioid ovarian cancers

<table>
<thead>
<tr>
<th>LOH on chromosome arm</th>
<th>6q</th>
<th>9p</th>
<th>11q</th>
<th>17p</th>
<th>17q</th>
<th>22q</th>
<th>TP53(^a) mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endometriosis</td>
<td>0/30</td>
<td>6/34</td>
<td>6/33</td>
<td>0/17</td>
<td>0/30</td>
<td>6/40</td>
<td>0/40</td>
</tr>
</tbody>
</table>

\(^a\) The top figure is the number of tumors with LOH at any marker on the indicated chromosome over the total number of informative tumors. The number in parentheses is the percentage of informative tumors showing LOH.

\(^b\) TP53 mutation was assessed by SSCP analysis of exons 5, 6, 7, and 8 in tumor and matched normal DNA. Two cases were sequenced and were found to contain missense mutations.

Table 3 Allelotype of endometriosis cases showing LOH

<table>
<thead>
<tr>
<th>Case</th>
<th>S171</th>
<th>S161</th>
<th>S1328</th>
<th>S1336</th>
<th>S304</th>
<th>PDGF ( \beta )</th>
<th>S302</th>
<th>CYP2D</th>
<th>S276</th>
<th>S274</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>LOH</td>
<td>LOH</td>
<td>NI</td>
<td>NI</td>
<td>LOH</td>
<td>NI</td>
<td>LOH</td>
</tr>
<tr>
<td>E5</td>
<td>LOH</td>
<td>LOH</td>
<td>NI</td>
<td>LOH</td>
<td>HET</td>
<td>NI</td>
<td>HET</td>
<td>LOH</td>
<td>NI</td>
<td>LOH</td>
</tr>
<tr>
<td>E7</td>
<td>LOH</td>
<td>LOH</td>
<td>HET</td>
<td>HET</td>
<td>LOH</td>
<td>NI</td>
<td>LOH</td>
<td>LOH</td>
<td>NI</td>
<td>LOH</td>
</tr>
<tr>
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<td>NI</td>
<td>HET</td>
<td>NI</td>
<td>HET</td>
<td>NI</td>
<td>HET</td>
<td>LOH</td>
<td>NI</td>
<td>HET</td>
</tr>
<tr>
<td>E15</td>
<td>NO</td>
<td>HET</td>
<td>NI</td>
<td>LOH</td>
<td>HET</td>
<td>LOH</td>
<td>HET</td>
<td>LOH</td>
<td>NI</td>
<td>HET</td>
</tr>
<tr>
<td>E23</td>
<td>NI</td>
<td>LOH</td>
<td>HET</td>
<td>LOH</td>
<td>NI</td>
<td>NI</td>
<td>HET</td>
<td>LOH</td>
<td>NI</td>
<td>HET</td>
</tr>
<tr>
<td>E24</td>
<td>LOH</td>
<td>LOH</td>
<td>HET</td>
<td>NI</td>
<td>NI</td>
<td>NI</td>
<td>HET</td>
<td>NI</td>
<td>NI</td>
<td>HET</td>
</tr>
<tr>
<td>E37</td>
<td>HET</td>
<td>HET</td>
<td>NI</td>
<td>LOH</td>
<td>HET</td>
<td>HET</td>
<td>HET</td>
<td>HET</td>
<td>NI</td>
<td>HET</td>
</tr>
<tr>
<td>E43</td>
<td>NI</td>
<td>LOH</td>
<td>HET</td>
<td>NI</td>
<td>NI</td>
<td>HET</td>
<td>HET</td>
<td>HET</td>
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<td>HET</td>
</tr>
<tr>
<td>E51</td>
<td>NI</td>
<td>LOH</td>
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<td>NI</td>
<td>HET</td>
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<td>HET</td>
<td>HET</td>
<td>HET</td>
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</tr>
<tr>
<td>E45</td>
<td>NI</td>
<td>HET</td>
<td>LOH</td>
<td>HET</td>
<td>NI</td>
<td>HET</td>
<td>HET</td>
<td>HET</td>
<td>HET</td>
<td>HET</td>
</tr>
<tr>
<td>E54</td>
<td>NI</td>
<td>HET</td>
<td>LOH</td>
<td>HET</td>
<td>NI</td>
<td>HET</td>
<td>HET</td>
<td>HET</td>
<td>HET</td>
<td>HET</td>
</tr>
</tbody>
</table>

\(^a\) HET, heterozygous; NI, not informative.
abnormalities by SSCP and microsatellite analysis would only be possible if these abnormalities were monoclonal. Additionally, a monoclonal origin would be expected if endometriotic lesions developed by clonal expansion of a genetically altered precursor cell, as has been demonstrated in the majority of cancers (27, 28). Only one study has addressed this question in endometriosis, and although the authors concluded that the majority were monoclonal, this was based on only five samples, three of which were monoclonal (22). We have confirmed this finding by demonstrating monoclonality in 14 of 17 informative cases. Although this is consistent with the multistep model for carcinoma development, it does not necessarily mean that endometriotic lesions are inherently premalignant because a monoclonal pattern has been observed in both normal and benign structures (29, 30). In fact, when we examined normal endometrial glands, we found that these too were predominantly monoclonal, demonstrating that in this respect, endometriotic cysts are not abnormal. With regard to the origin of the ectopic endometrium, a monoclonal pattern could arise either by implantation of a single endometrial cell or by metaplasia of a single peritoneal cell. In the case of the polyclonal cysts, these may have arisen by a combination of both events or through the implantation of a polyclonal group of refluxed endometrial cells. Irrespective of the origin of the ectopic endometrium, the fact that the majority of implants are monoclonal enabled us to pursue a molecular genetic analysis with a reasonable expectation of detecting genetic abnormalities if indeed any existed.

We analyzed TP53 and RASK because mutations in these genes have been described in ovarian cancers. Consistent with the small study of Vercellini et al. (16), we did not detect mutations in either of these genes in endometriotic lesions. To identify other potential chromosomal regions involved in the development of endometriosis, we

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Fig. 5. Allelic deletion for endometrioid ovarian cancer OC95 and endometriosis cases E7, E23, and E21. □, no LOH; ■, LOH; ■, constitutional homozygosity. Left, the microsatellite markers used and their approximate locations. Right, for each informative locus, the autoradiography of the PCR result from the normal (N), tumor (T), and endometriosis (E) DNA is shown.
examined for LOH on chromosome arms that previous studies have identified as harboring TSGs of relevance in ovarian tumorigenesis (23–25, 31). No LOH was observed on chromosome arms 6q, 17p, or 17q, but 11 of 40 (28%) cases showed LOH at one or more loci on chromosome arms 9p, 11q, or 22q. Although this frequency of LOH is low compared with that observed in malignant ovarian tumors, it is remarkably high compared with benign or borderline ovarian tumors.

Candidate TSGs on 11q and 22q have not yet been identified in ovarian cancer, but evidence is accumulating that multiple TSGs reside on each of these chromosome arms. In the case of 9p21, LOH is remarkably high compared with benign or borderline ovarian tumors. It is frequently observed in early stage and low-grade tumors, although point mutations in CDKN2 are rare (31). It will be of some interest to determine if the target of the LOH on 9p in endometriosis is accompanied by CDKN2 mutations or if, as seems to be the case in ovarian cancers, another 9p gene is the target of the allelic losses.

This study has added weight to the body of evidence that points to an underlying genetic component in the development of endometriosis and raises some interesting new questions that should now be investigated: (a) it is not possible to determine from this study whether the LOH detected is targeting genes involved in the initiation of endometrioid proliferation or progression to more aggressive disease. Although the absence of LOH in the majority of endometriosis cases argues for a role only in progression, it does not preclude the possibility that more subtle genetic lesions are present in these samples; (b) if LOH is associated with progression to more aggressive disease and possibly to carcinoma, could this be used to identify women at higher risk of developing ovarian cancer. Resolution of this should be possible by genetic analysis of endometrioid carcinoma arising from within or adjacent to endometriotic lesions; and (c) we have examined only a few genetic loci in endometriosis based on LOH frequency in predominantly serous ovarian cancers. As highlighted by this and other studies (32), endometrioid ovarian cancers, which are possibly a more relevant model for endometrioid development, seem to differ in their LOH patterns, highlighting the need for an expanded allelotype study of both endometrioid and endometrioid ovarian cancers.

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