Molecular genetic evidence that endometriosis is a precursor of ovarian cancer


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Ovarian cancer ranks fourth in cancer deaths among women aged 45–64 years, and is the most lethal of all gynaecological cancers (http://seer.cancer.gov/). The high mortality rate arises mainly because the disease is asymptomatic in its initial stages, making its early detection difficult.1 At the time of diagnosis, dissemination has occurred in more than 70% of cases, at which point the 5-year survival rate is less than 20%. Ninety percent of all ovarian cancers are epithelial in origin, and are classified according to their cell types (serous, mucinous, endometrioid, clear cell and unclassified or mixed histology).1 Different etiological factors have been implicated in these subtypes2 although, at present, little is known about the molecular events involved in their individual development.

Endometrioid and clear cell subtypes have been associated with the benign disease, endometriosis. This is a complex genetic trait which affects up to 10% of women in their reproductive years.3 It causes pelvic pain, severe dysmenorrhea (painful periods) and cancers are epithelial in origin, and are classified according to which affects up to 10% of women in their reproductive years.3 It is a benign disease, endometriosis. This is a complex genetic trait involving common environmental, immunological, hormonal or genetic factors.17 Molecular genetic studies of allele loss have demonstrated a strong link between endometriosis and endometriosis-associated ovarian cancers (EOACs)—in particular, the endometrioid and clear cell subtypes. However, it is still unclear whether endometriosis is a precursor to EOACs, or whether there is an indirect link because similar factors predispose to both diseases. In order to search for evidence of clonal progression, we analyzed 10 EOACs (endometrioid = 4; clear cell = 6) with coexisting endometriosis for common molecular genetic alterations in both the carcinoma and corresponding endometriosis. We used 82 microsatellite markers spanning the genome to examine loss of heterozygosity (LOH) in the coexisting carcinoma and endometriosis samples. A total of 63 LOH events were detected in the carcinoma samples; twenty two of these were also detected in the corresponding endometriosis samples. In each case, the same allele was lost in the endometriosis and cancer samples. Interestingly, no marker showed LOH in the endometriosis alone. These data provide evidence that endometriosis is a precursor to EOACs.

Histopathology and epidemiology studies have consistently demonstrated that endometriosis is a precursor to EOACs—in particular, the endometrioid and clear cell subtypes. However, it is still unclear whether endometriosis is a precursor to EOACs, or whether there is an indirect link because similar factors predispose to both diseases. In order to search for evidence of clonal progression, we analyzed 10 EOACs (endometrioid = 4; clear cell = 6) with coexisting endometriosis for common molecular genetic alterations in both the carcinoma and corresponding endometriosis. We used 82 microsatellite markers spanning the genome to examine loss of heterozygosity (LOH) in the coexisting carcinoma and endometriosis samples. A total of 63 LOH events were detected in the carcinoma samples; twenty two of these were also detected in the corresponding endometriosis samples. In each case, the same allele was lost in the endometriosis and cancer samples. Interestingly, no marker showed LOH in the endometriosis alone. These data provide evidence that endometriosis is a precursor to EOACs.

Key words: endometrioid ovarian cancer; clear cell ovarian cancer; endometriosis; precursor lesions; loss of heterozygosity

Material and methods

Tissue samples and microdissection

Unlinked, anonymised, archival specimens from 10 women, with ovarian endometriosis and cancer (Table I; Fig. 1), were obtained from the John Radcliffe Hospital, Oxford, UK; Birmingham Women’s Hospital, Birmingham, UK; Fox Chase Cancer Center, Philadelphia, PA; and M.D. Anderson, Houston, TX. IRB approval to collect and evaluate tissue samples had been obtained from each participating institute. Histological sections were prepared from formalin-fixed, paraffin-embedded tissue. Sections were cut from blocks representing each lesion; one section was stained with hematoxylin and eosin (H&E) for review by a histopathologist, and 5 sections were lightly stained with hematoxylin and eosin.

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for microdissection. Each section was microdissected using either the Leica AS LMD system (Leica Microsystems, Wetzlar, Germany) or the PixCell II Laser Capture Microdissection system (Arcturus Engineering, Mountain View, CA), to separate the endometriosis, the carcinoma and surrounding normal stromal tissue. DNA extraction was performed using the Qiagen QIAamp DNA Micro kit, according to the manufacturer’s instructions (Qiagen, Crawley, Sussex, UK).

**Whole genome amplification**

Whole genome amplification (WGA) was performed to gain sufficient DNA for loss of heterozygosity (LOH) analysis, because only minute quantities of DNA could generally be obtained from the microdissected endometriosis and carcinoma samples. DNA was amplified using the GenomePlex WGA kit (Sigma-Aldrich, Dorset, UK), according to the manufacturer’s instructions. Whole genome amplified DNA was purified using the Qiagen MinElute PCR purification kit (Qiagen), prior to LOH analysis.

**LOH analysis**

Polymorphic microsatellite markers were chosen from all 22 autosomal chromosomes (The Wellcome Trust Centre for Human Genetics, Oxford or Applied Biosystems, Cheshire, UK). Owing to the poor quality of the DNA from our archival samples, the markers were chosen based on the small size (<200 bp) of the product. The following microsatellite markers were used: D1S230; D1S498; D1S213; D2S319; D2S165; D2S391; D2S112; D2S2330; D2S396; D2S125; D2S1266; D3S1547; D3S1271; D3S1614; D3S1311; D4S2935; D4S392; D4S407; D4S1586; D5S648; D5S407; D5S433; D6S470; D6S1568; D6S1627; D8S262; D8S1577; D7S2532; D7S494; D7S515; D8S264; D8S261; D8S270; D8S272; D9S285; D9S1877; D9S1766; D9S164; D10S548; D10S196; D10S537; D10S215; D10S587; D10S212; D11S4046; D11S4191; D11S1314; D11S1793; D12S83; D12S79; D12S1723; D13S175; D13S153; D13S1625; D13S158; D14S283; D14S275; D14S70; D14S63; D14S65; D15S1002; D15S987; D16S3075; D16S520; D17S799; D17S787; D17S785; D18S63; D18S452; D18S1102; D19S884; D19S221; D19S210; D20S178; D20S171; D21S1256; D21S266; D22S83 (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unists; http://www.genlink.wustl.edu/genethon_frame/). One oligonucleotide from each pair was fluorescently labeled with FAM, HEX or NED (The Wellcome Trust Centre for Human Genetics, Oxford or Applied Biosystems). PCR was performed in a 20 μl reaction mixture containing 1× Qiagen PCR Master Mix (Qiagen), 0.5 μM concentrations of each primer pair and 30 ng of DNA from the microdissected endometriosis, ovarian carcinoma or normal ovarian stromal tissue. PCR amplification was performed as follows: 95°C for 5 min, 35 cycles of 96°C for 1 min, an annealing step of 53°C for 1 min, 72°C for 1 min, followed by a final extension step of 72°C for 10 min 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).

**Table 1 – Clinical features of the 10 samples used in the analysis, including age of diagnosis, tumor histology, stage and site of the ovarian endometriosis in relation to adenocarcinoma.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Age</th>
<th>Carcinoma</th>
<th>Stage</th>
<th>Site of endometriosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>146</td>
<td>66</td>
<td>Endometrioid</td>
<td>1A</td>
<td>Adjacent</td>
</tr>
<tr>
<td>2165</td>
<td>54</td>
<td>Endometrioid</td>
<td>1B</td>
<td>Adjacent</td>
</tr>
<tr>
<td>914</td>
<td>48</td>
<td>Endometrioid</td>
<td>1B</td>
<td>Contralateral</td>
</tr>
<tr>
<td>247</td>
<td>41</td>
<td>Clear cell</td>
<td>1A</td>
<td>Adjacent</td>
</tr>
<tr>
<td>18542</td>
<td>53</td>
<td>Mixed, predominantly endometrioid with some clear cell foci</td>
<td>–</td>
<td>Adjacent</td>
</tr>
<tr>
<td>11M</td>
<td>60</td>
<td>Clear cell</td>
<td>1C</td>
<td>Adjacent</td>
</tr>
<tr>
<td>10M</td>
<td>32</td>
<td>Clear cell</td>
<td>Borderline</td>
<td>Adjacent</td>
</tr>
<tr>
<td>3870</td>
<td>77</td>
<td>Clear cell</td>
<td>1B</td>
<td>Contralateral</td>
</tr>
<tr>
<td>8872</td>
<td>61</td>
<td>Mixed, predominantly clear cell with some endometrioid foci</td>
<td>1A</td>
<td>Contralateral</td>
</tr>
<tr>
<td>9684</td>
<td>48</td>
<td>Clear cell</td>
<td>1C</td>
<td>Contralateral</td>
</tr>
</tbody>
</table>

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

**Results**

In order to search for evidence of clonal progression, we analyzed 10 EAOC cases with coexisting endometriosis (Fig. 1), for common molecular genetic alterations. LOH analysis used 82 microsatellite markers. At least one marker from all the autosomal chromosome arms, except 12p and 20p, were used in the analysis. Thirty-seven of the 82 markers showed LOH in at least 1 carcinoma sample. A total of 63 LOH events were detected in the carcinoma samples, and 22 of these LOH events were also detected in the corresponding endometriosis (Tables II and III; Fig. 2a). No markers showed LOH in the endometriosis alone, and all 22 LOH events involved the same allele in both the carcinoma and endometriosis (Table III and Fig. 2a). LOH at one or more markers showed LOH are shown. Common LOH events have been identified in endometriosis and in both endometrioid and clear cell ovarian tumors and in samples which are in the same and in contralateral ovaries.

In all 9 lesions in which LOH was detected, LOH was detected more frequently in the carcinoma than in the endometriosis (Table II; Fig. 2b). This suggests that the endometriosis samples were not contaminated with carcinoma, and is consistent with the fact that all our samples were obtained by laser microdissection. LOH in both the endometriosis and carcinoma was detected on chromosomes 3q, 4q, 5q, 6p, 7q, 10q, 11p, 11q, 13q, 14q, 16q, 17q, and 21q (Tables II and III). Additional LOH events were detected in the carcinomas alone on chromosomes 2p, 2q, 3p, 3p, 6p, 7p, 8q, 9p, 10q, 12q, 14p, 17p, 18p, 18q, 19p, 20q and 22q (Table II). DSS407 at 5q11.2, D6S470 at 6q24.3 and D16S520 at 10q23 showed LOH in 2 endometriosis/tumor pairs and D17S787 at 17q22-23.2 showed LOH in 3 endometriosis/carcinoma samples (Table III).

Assuming equal probabilities of loss of paternal and maternal alleles, the probabilities of losing the same allele for each LOH event is $p = 0.5$. Therefore, the probability of identical alleles being lost by chance in each case, was $p = 0.5$ for sample 2165, where there was one common LOH event; $p = 0.5^{2} = 0.25$ for samples 247, 10M, 8872 and 842, where there were 2 markers showing LOH of the same allele; $p = (0.5)^{3} = 0.125$ for sample 914, showing 3 LOH events in common; $p = 0.5^{4} = 0.0625$ for 18542, showing 4 events in common; and $p = 0.5^{5} = 0.03125$ for sample 11M, where there was one common LOH event; and $p = 0.5^{6} = 0.015625$ for sample 842, we were able to confirm LOH at D17S787 (data not shown).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Clinical details</th>
<th>LOH events/informative markers</th>
<th>Microsatellite marker and loci at which LOH was detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>146T</td>
<td>Endometrioid</td>
<td>1/19</td>
<td>D18S542 (18q12.2)</td>
</tr>
<tr>
<td>146E</td>
<td>Adjacent</td>
<td>0/39</td>
<td></td>
</tr>
<tr>
<td>2165T</td>
<td>Endometrioid</td>
<td>3/51</td>
<td>D2S319 (2p25); D7S515 (7q22); D8S272 (8q24)</td>
</tr>
<tr>
<td>2165E</td>
<td>Adjacent</td>
<td>1/28</td>
<td>D7S515 (7q22)</td>
</tr>
<tr>
<td>914T</td>
<td>Endometrioid</td>
<td>4/28</td>
<td>D5S407 (5q11.2); D9S285 (9p23-22); D13S175 (13q11); D16S520 (16q24)</td>
</tr>
<tr>
<td>914E</td>
<td>Contralateral</td>
<td>3/10</td>
<td>D5S407 (5q11.2); D13S175 (13q11); D16S520 (16q24)</td>
</tr>
<tr>
<td>247T</td>
<td>Clear cell</td>
<td>11/39</td>
<td>D3S111 (3q26-27); D5S658 (5p14); D8S433 (8q21); D6S1568 (6p21); D7S494 (7p11-11.1); D9S285 (9p23-22); D10S548 (10p14-12); D10S215 (10q23); D13S265 (13q31); D14S283 (14q11); D14S275 (14q12)</td>
</tr>
<tr>
<td>247E</td>
<td>Adjacent</td>
<td>2/45</td>
<td>D5S433 (5q21); D13S265 (13q31)</td>
</tr>
<tr>
<td>18542T</td>
<td>Adjacent</td>
<td>11/49</td>
<td>D5S407 (5q11.2); D5S433 (5q21); D6S470 (6p24.3); D6S1627 (6p14-15); D10S537 (10q23); D11S4046 (11p15.5); D11S4191 (11p11.2); D11S1314 (11q13.1); D13S265 (13q31); D17S787 (17q22-23.2); D18S542 (18p11)</td>
</tr>
<tr>
<td>18542E</td>
<td>Adjacent</td>
<td>4/51</td>
<td>D6S470 (6p24.3); D10S537 (10q23); D11S4191 (11p11.2); D17S787 (17q22-23.2)</td>
</tr>
<tr>
<td>11MT</td>
<td>Clear cell</td>
<td>9/44</td>
<td>D3S1614 (3q26.3); D3S1311 (3q26.32-3q27); D6S470 (6p24.3); D10S537 (10q22.1); D10S212 (10q26); D13S265 (13q31); D17S787 (17q22-23.2); D18S452 (18p11)</td>
</tr>
<tr>
<td>11ME</td>
<td>Adjacent</td>
<td>6/39</td>
<td>D3S1614 (3q26.2); D3S1311 (3q26.32-3q27); D6S470 (6p24.3); D5S407 (5q11.2); D10S537 (10q22.1); D13S265 (13q31); D17S787 (17q22-23.2)</td>
</tr>
<tr>
<td>10MT</td>
<td>Clear cell</td>
<td>4/43</td>
<td>D4S407 (4q24); D14S283 (14q11.1-q12); D19S884 (19p13); D21S1256 (21q22)</td>
</tr>
<tr>
<td>10ME</td>
<td>Adjacent</td>
<td>2/41</td>
<td>D4S407 (4q24); D21S1256 (21q22)</td>
</tr>
<tr>
<td>3870T</td>
<td>Clear cell</td>
<td>0/16</td>
<td>–</td>
</tr>
<tr>
<td>3870E</td>
<td>Contralateral</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td>8872T</td>
<td>Clear cell</td>
<td>15/46</td>
<td>D2S319 (2p25); D2S2330 (2q12-13); D3S1311 (3q26.32-3q27); D5S407 (5q11.2); D10S537 (10q23); D10S215 (10q23); D11S4046 (11p15.5); D11S1314 (11q13.1-q14.3); D12S797 (12q24.21); D13S265 (13q31.2-q31.3); D14S65 (14q32); D17S787 (17p12); D17S785 (17q5); D19S221 (19p13.2-13.3); D20S171 (20p13)</td>
</tr>
<tr>
<td>8872E</td>
<td>Contralateral</td>
<td>2/51</td>
<td>D11S1314 (11q13.1-q14.3); D14S65 (14q32)</td>
</tr>
<tr>
<td>842T</td>
<td>Clear cell</td>
<td>5/47</td>
<td>D3S1286 (3pter-3p24.2); D3S1311 (3q26.32-3q27); D5S507 (5q11.2); D12S1723 (12q13); D17S787 (17q22-23.2)</td>
</tr>
<tr>
<td>842E</td>
<td>Contralateral</td>
<td>2/40</td>
<td>D5S507 (5q11.2); D17S787 (17q22-23.2)</td>
</tr>
<tr>
<td>All T</td>
<td></td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>All E</td>
<td></td>
<td>22</td>
<td></td>
</tr>
</tbody>
</table>

Clinical details (histology of the ovarian tumor and the site of the endometriosis in relation to the tumor) and the loci of the markers which showed LOH are shown. Common LOH events have been identified in endometriosis and in both endometrioid and clear cell ovarian tumors and in samples which are in the same and in contralateral ovaries.
triosis and carcinoma, in all the common 22 LOH events combined is therefore very small ($p = 2.38 \times 10^{-2}$). In addition, this calculation has taken into account neither the probability of LOH occurring at the same locus in the endometriosis and cancer, nor that the regions of LOH in at least one case appeared to be the same (sample 11M between D3S1614 and D3S1311). The 2 cases where the endometriosis was located adjacent to the tumor (1 case of endometrioid (18542) and 1 case of clear cell ovarian cancer (11M)) showed the highest number of common LOH levels, but even case 914, where the endometriosis and tumor were in separate ovaries, showed 3 events in common (Table II). We conclude that endometriosis is likely to be a genetic precursor to EAOC in these 8 cases.

### Discussion

In order to search for evidence of clonal progression, we analyzed 10 EAOC cases with coexisting endometriosis for common molecular genetic alterations. Using 82 microsatellite markers spanning the genome, we detected 63 LOH events in the carcinoma samples; twenty two of these were also detected in the corresponding endometriosis sample, involving the same allele in each case. Interestingly, no marker showed LOH in the endometriosis alone. Statistical analyses suggest that it is very unlikely that these common genetic alterations have occurred as independent events, and therefore we have provided evidence that endometriosis can be a clonal precursor to EAOCs.

Although endometriosis is a "benign" disease, it has many characteristics of neoplasia. Endometriosis may show unrestrained growth and increased vascularization, and even features classically associated with malignancy, such as tissue invasion and metastasis. By detecting LOH in endometriosis lesions, we have also provided molecular evidence to suggest that endometriosis lesions can be true neoplasms, that is, they derive from a clonal proliferation of cells arising from genetic changes, which provide that clone with a replicative advantage.

There have only been a few LOH studies analyzing solitary endometriosis and EAOC. For example, Jiang et al. (1998) ana-
lyzed 14 cases of endometriosis synchronous with endometrioid ovarian cancer for LOH on 12 chromosome arms, using 25 markers. In 9/11 cases where the carcinoma had either arisen within, or was adjacent to the endometriosis, LOH of the same allele was detected. However, none of the 3 cases where the endometriosis and carcinoma were from contralateral ovaries displayed

**FIGURE 2**

(a) LOH at markers D7S515, D5S407, DSS433, D105-215, D17S787 and D6S470 in both ovarian carcinoma and corresponding endometriosis from cases 2165, 914, 18542, 18542, 18542 and 11M respectively. (b) LOH at markers D5S407, D11S4046, D18S452, D19S221, AFM0860wx9 and D3S1311 in the carcinoma alone, with retention in the corresponding endometriosis, in cases 18542, 8872, 11M, 8872, 247 and 8872, respectively. Representative electropherograms from the analysis of normal ovarian stroma (N), carcinoma (CA) and coexisting endometriosis (E) are shown. The arrow indicates the ‘lost’ allele in each case. Sizes of the alleles, in base pairs, are indicated under the allele peak.
any common LOH, and in one of these cases LOH was detected in the endometriosis and not in the carcinoma. However, the majority of LOH studies have been limited by a number of factors, including (i) the small number of markers analyzed (ii) not using laser microdissection and (iii) nonquantitative LOH analysis from autoradiographs. We have improved upon these studies, reducing the potential for artifacts, by using fine scale microdissection and quantitative, fluorescent-based detection of LOH. The sample size in our study, and others, is small: Histopathologists diagnosing ovarian cancer often do not look for, or note, the presence of endometriosis, and therefore the samples are hard to obtain. However, by using a larger panel of markers, we were able to detect many more LOH events common to both the carcinoma and endometriosis, providing more convincing evidence of a common lineage and demonstrating that common allele loss is not a chromosomespecific event. In addition, to our knowledge, this study is the first to show evidence that endometriosis can be a clonal precursor to (i) clear cell, as well as endometrioid ovarian cancer and (ii) carcinoma from contralateral ovaries, as well as cases where the endometriosis is adjacent to the carcinoma.

The frequency of malignant transformation in ovarian endometriosis is currently uncertain. Large pathology series have found ovarian cancer in 5–10% of endometriomas, and malignant transformation of endometriomas has been estimated as 0.7–1.6% over an average of 8 years. Other studies have found endometriosis in 21–54% of endometrioid and clear cell ovarian carcinomas, but this is likely to be an underestimate, as the cancer can obliterate any endometriosis present. It may well be that the majority, if not all, of endometrioid and clear cell ovarian carcinomas originally derive from endometriosis, although there is currently no evidence for this.

Some studies have suggested that EAOCs may be a separate entity from other ovarian carcinomas, where patients with EAOC are generally premenopausal, and have early stage disease and better survival. These clinical observations could be explained by the fact that the carcinoma may be detected at an earlier stage because of endometriosis-associated symptoms, or that the endometriosis could be obliterated by the time the disease has progressed to the higher stages. In addition, high stage EAOCs have been documented. All our samples were low stage, and it would certainly be interesting to analyze high stage tumors coexisting with endometriosis to determine whether there were common molecular alterations. Since the two diseases share the same predisposing factors, and biological consequences of endometriosis, such as infertility, are associated with increased risks of ovarian cancer, we cannot conclude that endometriosis is a genetic precursor to EAOCs in all cases. Unfortunately, we do not have data as to whether our patients had a long history of infertility or not. Clearly, however, future studies not looking for genes involved in EAOCs will have significance in the early development of at least a subset of endometrioid and clear cell ovarian cancers.

The ultimate aim of our studies is to identify genes that are involved in the development of endometriosis and its progression to malignancy. It has been shown that LOH is selected for, and therefore are detected at high frequencies, when it is involved in the inactivation of tumor suppressor genes. In our study, LOH was generally detected at low frequencies; however, DSS407 at 5q11.2, D6S470 at 6p24.3 and D13S265 at 13q31.2-q31.3 showed LOH in 2 endometriosis/tumor pairs and D17S787 at 17q22.2 showed LOH in 3 endometriosis/carcinoma samples, and therefore these loci may contain candidate tumor suppressor genes involved in the initiation of EAOCs. Our findings suggest that larger future studies using a similar strategy with a higher density of polymorphic markers will provide an opportunity to identify loci involved in clonal progression of endometriosis to cancer.

An increased understanding of the molecular events involved in the initiation and development of EAOC will provide a basis for developing novel forms of early diagnosis and therapy. Our findings have provided convincing evidence that endometriosis can be a precursor to EAOCs and further studies are needed to establish how molecular markers of progression can be used to identify women with endometriosis at highest risk of EAOC.

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References