Oncogenic events associated with endometrial and ovarian cancers are rare in endometriosis

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ABSTRACT: Endometriosis displays some features that resemble malignant processes, including invasive growth, resistance to apoptosis and distant implantation. The objective of this study was to investigate whether gene alterations that are frequent in endometrial and/or ovarian cancers contribute to the pathogenesis of endometriosis. Biopsies were obtained from ectopic endometriosis lesions from 23 patients with revised American Fertility Score stage 1 (n = 1), 2 (n = 10), 3 (n = 11) or 4 (n = 1) endometriosis. Six genes (APC, CDKN2A, PYCARD, RARB, RASSF1 and ESR1) were analyzed for promoter hypermethylation using methylation-specific melting curve analysis, and 9 genes (BRAF, HRAS, NRAS, CTNNB1, CDK4, FGFR3, PIK3CA, TP53 and PTEN) were analyzed for mutations using denaturing gradient gel electrophoresis and direct sequencing. An oncogenic mutation in KRAS (c.34G > T; p.G12C) was detected in a single lesion. No gene alterations were found in the remaining samples. Our data suggest that genetic and epigenetic events contributing to endometrial and ovarian cancers are rare in endometriosis. However, other proto-oncogenes and tumor suppressor genes should be tested for alterations in order to identify the molecular basis of the susceptibility of endometriosis to malignant transformation.

Key words: endometriosis / endometrium / malignancy / mutation / methylation

Introduction

Endometriosis is defined as the presence and growth of endometrial-like tissue outside the uterus (Giudice and Kao, 2004). This painful disease affects 6–10% of all women and frequently causes infertility. The much debated pathogenesis involves retrograde menstruation as well as numerous incompletely characterized hormonal, immunological and genetic factors. Some features of endometriosis resemble cancer, such as local invasive growth, resistance to apoptosis and distant implantation (Nezhat et al., 2008). Moreover, endometriosis occasionally transforms into endometrial cancer (Prowse et al., 2002; Barton et al., 2008). In a previous study, the CDKN2A promoter was hypermethylated in 1 of 46 cases of endometriosis (Martini et al., 2002), but the methylation status of the remaining genes has, to our knowledge, not been studied in this disease. We also analyzed the same endometriosis DNA samples for mutations in proto-oncogenes and tumor suppressor genes that are frequently mutated in endometrial and ovarian carcinomas, including PTEN, TP53, BRAF, CTNNB1, CDK4, FGFR3, PIK3CA, HRAS, KRAS and NRAS (Forbes et al., 2008). Our investigations suggest that mutation and hypermethylation events in these genes are rare in endometriosis.
Materials and Methods

DNA was extracted from ectopic endometriosis lesions (Ec) from 23 women with American Fertility Score stage 1 (n = 1), 2 (n = 10), 3 (n = 11) or 4 (n = 1) (samples described by Vestergaard et al., 2010; Vestergaard et al., 2011). The mean age (±SD) of the participants was 31.0 (±5.2) years. The methylation status of promoter CpG islands was analyzed using methylation-specific melting curve analysis (MS-MCA). This method resolves differences in methylation status on the basis of differences in melting temperature after treatment of DNA with sodium bisulfite, which converts unmethylated cytosine to uracil but leaves methylated cytosine unchanged (Worm et al., 2001). Primers were designed to be specific for bisulfite-converted DNA and to not discriminate between methylated and unmethylated alleles. In brief, 500 ng of genomic DNA was treated with sodium bisulfite, using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, USA). MS-MCA was carried out using the LightCycler 2.0 instrument (Roche, Basel, Switzerland) and the FastStart DNA Master SYBR Green I Kit (Roche). CpGenome Universal Methylated DNA (Chemicon, Billerica, MA, USA) was used as a methylated control, and DNA extracted from peripheral blood lymphocytes from a healthy donor was used as an unmethylated control. A 1:1 mixture of these controls was used to exclude bias for each PCR assay. In our hands, MS-MCA detects methylated DNA within a background of unmethylated DNA at a sensitivity of 1–2%. Primers and conditions are listed in Supplementary data, Table S1.

Mutations were detected using PCR in combination with denaturing gradient gel electrophoresis (DGGE; Myers et al., 1987). PCR was performed in 15 μl reactions containing 1 unit of HotStarTaq Plus DNA Polymerase (Qiagen, Hilden, Germany), 0.2 mM each dNTP, 12% sucrose, 10 pmol of each primer, 100 μM of each dNTP and 20 ng of DNA. The PCR products were analyzed in double-gradient denaturing gels (Cremonesi et al., 1997). Gels were then stained with ethidium bromide, and photographed under UV transillumination. In our hands, DGGE has a sensitivity of ~5% and thus is better suited for detection of low-abundant mutant DNA than standard Sanger sequencing. Primers and conditions are listed in Supplementary data, Table SII.

Results

The methylation status of the promoter regions of APC, CDKN2A, TIM31, RARB, RASSF1A and ESR1 was examined using MS-MCA. For all six promoter regions, the melting profile of DNA from the 23 endometriosis samples was similar to that of the unmethylated control, suggesting that silencing of these genes by promoter methylation is an infrequent event in endometriosis (see Fig. 1A for examples).

Next, DNA from endometriosis lesions was analyzed for mutations in PTEN (exons 1–9) and the mutation hot spots of TP53 (exons 5–9), BRAF (exons 11 and 15), CTNNB1 (exon 3), CDK4 (exon 2), FGFR3 (exons 7, 10 and 15) and PIK3CA (exons 9 and 20) and HRAS, KRAS, and NRAS (exons 2 and 3). DNA from tumor specimens or cell lines with known mutations was used as positive controls. One of the endometriosis lesions (Ec28, patient with stage 3 endometriosis) showed an aberrant band pattern for exon 2 of KRAS (Fig. 1B). Direct sequencing of DNA from this sample did not reveal any sequence alterations (data not shown). However, sequencing of DNA enriched for mutant DNA by excision of aberrant bands from the gel revealed a GGT to TGT transversion in codon 12 of KRAS, leading to substitution of a glycine residue with cysteine (c.34G → T; p.G12C; Fig. 1C). Repeated DGGE analysis and sequencing confirmed that the KRAS mutation in this sample was genuine and not a PCR artifact. The same aberrant bands could also be detected in the eutopic endometrium from the same patient, but the fraction of mutant DNA was clearly lower than in the endometriosis sample and close to the detection limit of the assay (Fig. 1B). This mutation was not found in blood from the same patient. No other mutations were found in the 23 endometriosis lesions.

Discussion

Systematic analysis of endometriosis lesions for genetic and epigenetic events implicated in the development of endometrial and ovarian cancers led to the identification of the KRAS p.G12C mutation in one sample. The RAS genes are potent oncogenes that have been unequivocally implicated as driver genes in the development of human malignancies. Mutations in codons 12, 13 and 61 of KRAS, including the G12C mutation described here, have been found at high frequencies in several types of cancer (Forbes et al., 2008). However, like other oncogenes that deliver strong mitogenic signals, oncogenic RAS is a potent inducer of senescence in primary cells (Serrano et al., 1997). Although the mechanism of oncogene-induced senescence is not fully understood, it may be due to unbalanced DNA replication that initiates a classic DNA-damage response (Bartkova et al., 2006). The presence of cells with an oncogenic KRAS mutation in an endometriosis lesion showing no evidence of cancer suggests that endometriosis cells may be more resistant to oncogene-induced senescence than normal cells, which could explain why endometriosis lesions are prone to carcinogenesis. However, further studies will be needed to address this theory.

Previous studies have shown that mutations in KRAS, TP53 and PTEN are frequent in endometriosis-associated carcinomas but absent in paired endometriosis tissues (Amemiya et al., 2004; Nezhat et al., 2008). Our study supports and extends these findings by showing that mutations in these genes and other genes frequently mutated in endometrial and ovarian cancers are rare in endometriosis. Furthermore, we have shown that genes that are frequently silenced by promoter hypermethylation in endometrial and ovarian cancers are unmethylated in endometriosis. As our methods for mutation and methylation analysis detect aberrant DNA in a background of wild-type DNA at a sensitivity of 2–5%, we cannot exclude that genetic and epigenetic alterations present at lower abundance could contribute to the development of endometriosis. Nevertheless, other proto-oncogenes and tumor suppressor genes should be tested for alterations in order to identify the molecular basis of the susceptibility of endometriosis to malignant transformation. One candidate gene is the tumor suppressor Lkb1. In a recent study, mice genetically engineered to inactivate Lkb1 only in the endometrial epithelium underwent malignant transformation of their entire endometrium with rapid extraterine spread and death, suggesting that inactivation of Lkb1 was sufficient to drive invasive endometrial cancer (Contreras et al., 2010).

Authors’ roles

A.L.V., U.B.K., P.G., and P.M.M. substantially contributed to the conception and design of the project, while all authors contributed to acquisition, analysis and interpretation of data. A.L.V., P.G. and
P.M.M. wrote the article, while K.T, U.B.K., T.M., H.R. and J.B.P. participated in critically revising it. All authors approved the final version of the article.

**Supplementary data**
Supplementary data are available at http://molehr.oxfordjournals.org/.

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