CLINICAL STUDY

# Screening for Ovarian Cancer in Women With Endometriosis by Using Fanconi DNA Repair Pathway

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## Abstract

**Objective:** Cells at endometriotic foci sometimes exhibit genomic instability, which allows them to break chromosomes and behave as neoplastic cells. In order to reach genomic instability, the cell has to undergo DNA damage. Cells can achieve this by several mechanisms, including knocking out one of the 6 major DNA repair systems such as Fanconi anemia (FA) pathway. Fanconi anemia is a rare genetic disorder characterized by skeletal anomalies, progressive bone marrow failure, cancer susceptibility and cellular hypersensitivity to DNA cross-linking agents, such as mitomycin C and cisplatin.
**Materials and Methods:** Seeking evidence of FA protein dysfunction in women with endometriosis, we screened ovarian surface epithelial cells from primary cultures established from 8 patients using hypersensitivity assays with cross-linking agents. In chromosomal breakage assays, normal ovarian epithelial control cells were mitomycin C (MMC) resistant, but three of the eight samples (two with stage 4 endometriosis and one with stage 3 disease) were hypersensitive. FANCD2 protein expression was reduced in these 3 cases. Lymphocytes from all eight patients were MMC resistant. Ectopic expression of normal FANCD2 cDNA increased FANCD2 protein and induced MMC resistance in the three hypersensitive lines tested.
**Results:** In some women with endometriosis tissue-restricted hypersensitivity to cross-linking agents is a frequent finding, and chromosomal breakage responses to MMC may be a sensitive screening strategy to identify women with endometriosis who may be at risk of developing ovarian cancer because of the antedating cytogenetic instability.
**Discussion:** Genetic or epigenetic events that result in tissue-specific FANCD2 gene suppression may represent a cause of this instability.

**Keywords:** endometriosis, Fanconi anemia, FANCD2, ovarian cancer

## Özet

### Fanconi DNA Onarım Yolu Kullanılarak Endometriyozisli Kadınlarda Yumurtalık Kanseri Taraması

**Amaç:** Endometriyotik odaklardaki hücrelerde bazen genomik dengesizlikler saptanabilmekte; bu durum kromozomların kırılmasına ve neoplastik hücreler gibi davranmalarına neden olabilmektedir. Genomik dengesizliğe ulaşılabilmesi için hücrenin DNA bozukluklarını tolere edebilmesi gerekir. Hücreler bunu farklı birçok mekanizmayla başarabilirler. Bunlardan biri, Fanconi anemi (FA) yolu gibi mevcut 6 DNA onarım sisteminden birini devre dışı bırakmaktır. Fanconi anemisi, ender bir genetik bozukluktur ve klinikte iskelet anomalileri, ilerleyici kemik iliği yetersizliği, kansere yatkınlık ve mitomisin-C ve sisplatin gibi DNA çapraz bağlayıcı ajanlara karşı hücresel aşırı duyarlılık şeklinde kendini gösterir.
**Materyal ve Metot:** Endometriyozisi olan kadınlarda FA protein disfonksiyonuna dair araştırmalar yapılırken, 8 hastada (primer kültürlerinde çapraz bağlayıcı hipersensitivite testleri kullanılarak) over yüzeyi epitelyal hücreleri taranmıştır. Kromozomal kırılma testlerinde, sağlıklı ovaryen epitelyal kontrol hücreleri mitomisin-C'ye (MMC) dayanıklıydı. Ancak, 8 örneğin 3'ünde (ikisi IV. evre ve biri III. evre endometriyozis idi) aşırı duyarlıydı. FANCD2 protein ekspresyonu bu üç hastada azalmıştı. Sekiz hastanın hepsinde lenfositler MMC'ye dayanıklıydı. Normal FANCD2 cDNA'nın ektoptik ekspresyonu, FANCD2 proteininin artmasına ve test edilen 3 hipersensitif seride MMC dayanıklılığının artmasına sebep oldu.

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**Sonuçlar:** Dolayısıyla, endometriyozisi olan bazı kadınlarda çapraz bağlayıcı ajanlara karşı aşırı duyarlılık sık görülen bir bulgudur. Ayrıca, over kanseri riski taşıyan endometriyozisli kadınları belirlemek için MMC'ye kromozomal kırılma yanıtı, duyarlı bir tarama stratejisi olarak kullanılabilir; bu şekilde belirlenen sitogenetik tutarsızlıklar kanser başlangıcına işaret edebilir.

**Tartışma:** Doku spesifik FANCD2 gen baskılanması ile sonuçlanan genetik veya epigenetik olaylar bu tipteki bir tutarsızlığın sebebi olarak görülebilir.

**Anahtar sözcükler:** endometriyozis, Fanconi anemisi, FANCD2, over kanseri

## Introduction

Endometriosis is defined as the implantation of endometrium-like glandular and stromal cells outside their normal location in the uterus. The pathogenesis implies a neoplastic process with loss of control of cell proliferation and local and distant spread; however, unlike cancer, endometriosis does not cause catabolic disturbance, metabolic consequences, or death (1). While endometriosis is not considered a premalignant condition, epidemiological, histopathological, and molecular data suggest that endometriosis does have neoplastic potential.

Two main hypotheses may explain an association between endometriosis and ovarian cancer. The first implicates the malignant transformation of endometriotic implants and the second maintains that both endometriosis and cancer share similar mechanisms or predisposing factors with a divergence in downstream pathways (2). The prevalence of endometriosis in patients with epithelial ovarian cancer, especially in endometrioid and clear cell types, has been confirmed to be higher than in the general population. While there is contradictory data on increased risk of developing any cancer in the lifetime of women with endometriosis(1), the largest population study from Sweden shows a 4.2-fold increased risk of ovarian cancer in endometriosis patients (3).

Cancer cells are often defective in one of the 6 major DNA repair pathways: mismatch repair, base-excision repair, nucleotide-excision, homologous recombination, non-homologous end-joining, and translesion synthesis. The study of inherited DNA repair disorders, such as Fanconi anemia, has yielded new insights to drug sensitivity and treatment of sporadic cancers, such as breast or ovarian epithelial tumors, in the general population. Specifically, our group has recently detected Fanconi phenotype in non-cancerous ovarian surface epithelial cells (OSE) from members of high-risk families showing excessive chromosomal breakage after exposure to DNA-damaging mitomycin C (MMC) and significantly reduced levels of the protein FANCD2 (4).

Twelve FA genes have been identified to date: FANCA, -B, -C, -D1, -D2, -E, -F, -G, -J, -L, M and -N (5-7). Of these, FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL, and FANCM form a nuclear core complex. While the functional scope of this complex has not been fully defined, it is clear that it must be completely intact to facilitate the monoubiquitination of the downstream FANCD2 protein (8). This modification permits FANCD2 to co-localize with BRCA1, BRCA2, and RAD51 in damage-induced nuclear foci (9, 10). The monoubiquitinated form (FANCD2-L; 162 kDa) can be readily identified and distinguished from the non-ubiquitinated form (FANCD2-S; 155kDa) by immunoblotting (11). Therefore, the MMC hypersensitive phenotype, which can be caused either by failure of cells to ubiquitinate FANCD2 or by loss of FANCD2 expression, can be readily screened using this paradigm.

In this study, we sought to identify abnormalities in responses to cross-linking agent and FA protein function in endometriotic lesions from endometriotic foci at the ovaries removed during therapeutic or diagnostic laparoscopic surgery. We report here that 3 of the 8 samples, 2 with stage 4 endometriosis (cases 3 and 5) and one with stage 3 disease (case 6) exhibited MMC-induced cytogenetic instability and reduced FANCD2 protein levels and that lymphocytes from none of these patients exhibited such genetic instability.

## Materials and Methods

Endometriotic samples were collected intraoperatively during laparoscopic surgery for treatment of endometriosis and after having obtained signed informed consent (OHSU IRB #8178, Mount Sinai Hospital IRB 99-552). The endometriotic foci were removed for diagnostic and therapeutic purposes using CO2 laser or endoshears. A total of 14 samples from 10 patients were obtained. The primary cultures were successfully obtained in 8 of the 10 patients. Two patients had stage 4 endometriosis (cases 3 and 5), and one patients (case 6) had stage 3 disease. The remaining 5 patients had disease limited to the ovaries. The control group consisted of 9 patients who underwent gynecologic surgery for a benign cause, e.g., uterine fibroids (4).

### Cell culture

A total of 14 samples were obtained from 10 patients including ovarian tissue (10 samples), peritoneal implants (2 samples), bowel serosal implant (one sample) and diapharagmatic implant (one sample). The successful primary short-term cultures were obtained from 8 of the 10 ovarian samples. The peritoneal, bowel, and diaphragmatic implants as well as 2 primary ovarian samples did not yield successful cultures, however DNA was extracted for future studies. The endometriotic implants were carefully removed laproscopically using CO2 laser for therapeutic and diagnostic purposes, placed in RPMI 1640 medium

and then enzymatically disaggregated with Collagenase I (GIBCO-Invitrogen, Grand Island, NY) for 4 hours. The cells were washed in RPMI 1640 medium (GIBCO-Invitrogen) and plated in 25 $cm^2$ flasks in RPMI 1640 supplemented with 20% FCS (Hyclone, Logan, UT), 10 µg/ml insulin (Sigma, St. Louis, MO) and 10 ng/ml EGF (R&D Systems, Minneapolis, MN). Ovarian cells were transformed by transduction with a retrovirus expressing SV40 large T-antigen (12), then transduced with pMMP retroviral vectors containing full-length FANCD2 cDNA (13,14). Lymphocytes were isolated from peripheral blood using Ficoll-Paque PLUS (Amersham Biosciences, Piscataway, NJ), then stimulated with 1% phytohemagglutinin (PHA) with and without MMC for 4 days before harvest. Harvested lymphocytes were used to prepare protein lysates and RNA, and for chromosomal breakage analyses.

**Chromosomal breakage analysis**

For breakage studies, cell cultures were incubated with 40 ng/ml MMC and 200 ng/ml diepoxybutane (DEB) at 37°C for 48 hours in RPMI 1640 medium in the dark. The cultures were then harvested with Colcemid, treated with hypotonic solution (0.075M KCI), the cells were fixed, stained with Wright's stain, and scored for chromosomal breaks and radial forms.

**Immunoblots**

$1x10^6$ cells were treated with or without 50 nM MMC for 48 hours. Whole-cell lysates and FANCD2 immunoblots were prepared as described elsewhere (4). Primary antibodies were monoclonal anti-FANCD2, diluted 1:200 (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-α tubulin, diluted 1:500 (Sigma); Secondary antibodies (1:10000 dilution) were horseradish peroxidase-conjugated goat anti-mouse or goat-anti-rabbit antibody (Bio-Rad, Hercules, CA).

DNA copy number analysis of FA and other chromosome instability genes.

The oligonucleotide array comparative genomic hybridization (oa-CGH) method used here has been described previously (15). Briefly, a whole-genome array with a 6-kb median probe spacing was used to map single and multiple copy number genomic alterations. Oligonucleotide probes (45-85 nucleotides in length) were tiled through genic and inter-genic regions. Genomic DNA extracted from primary OSE cells was fragmented to 500-2000 bp, then labeled with fluorescent dyes (15). Differentially labeled test and reference sample were combined in NimbleGen Hybridization Buffer (NimbleGen Systems, Madison, WI), denatured at 95°C for 5 minutes, hybridized for 18 hours at 42°C, arrays were washed with NimbleGen Wash Buffer System and dried. The reference sample was a pool of DNA (extracted from peripheral blood lymphocytes) from 6 male individuals (Promega, Madison, WI). Arrays were scanned using a GenePix 4000B scanner (Axon Instruments, Molecular Devices Corp., Sunnyvale, CA). The log2 ratios were averaged with a fixed window size corresponding to 5X, 10X, and 20X the median probe spacing. Log2 ratios were used as input to the DNA copy package of the Bioconductor software to produce the final segmentations (15) that demarcate DNA copy number changes.

## Results

**MMC-induced chromosomal breakage**

Epithelial cells from 9 control normal ovarian samples showed levels of MMC- and DEB-induced radial formation consistent with the range defined for other well-studied normal cell types (<20% of metaphases). The 9 samples were obtained from women undergoing gynecologic surgery for benign reasons, such as fibroid uterus and were published elsewhere (4). Three of the 8 endometriotic samples had increased levels of chromosomal breakage and radial formation. Peripheral blood lymphocytes from the same patients was obtained and subjected to MMC/DEB-induced chromosomal-breakage testing. None of the lymphocytes were hypersensitive to MMC or DEB.

**FA pathway defects identified by FANCD2 immunoblot**

Having determined that epithelial cells from the 3 patients with grade 3 and 4 endometriosis (cases 3, 5, and 6) exhibited cross-linking agent hypersensitivity, we sought to determine whether MMC/DEB sensitivity could be the result of inherited or acquired dysfunction of the FA proteins.

Eight primary cell cultures were treated with 50 nM MMC for 48 hours, then screened for the presence of FANCD2 long (L) and short (S) forms by immunoblotting (11). Reduced levels of FANCD2 protein (both the -L and -S forms) were consistently found in 3 breakage-positive samples, but not in

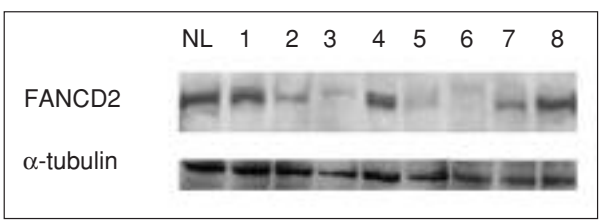


**Figure 1a.** FANCD2 immunoblots of 8 primary ovarian epithelial cells from endometriotic foci NI is normal control, #1-8 are the endometriotic ovarian samples. Cases 3, 5 and 6 show reduced levels of FANCD2 protein. A-tubulin loading control is at the bottom.

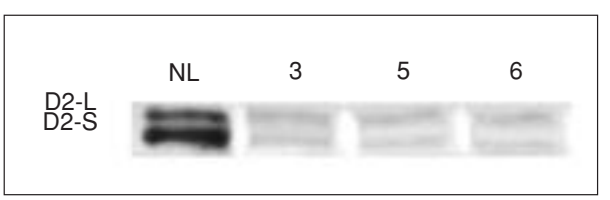


**Figure 1b.** Immunoblots of primary ovarian epithelial cells from endometriotic foci with reduced levels of FANCD2 protein. All 3 cases show ability to ubiquitinate FANCD2 as illustrated by the presence of FANCD2-L. FANCD2-S and FANCD2-L are indicated.

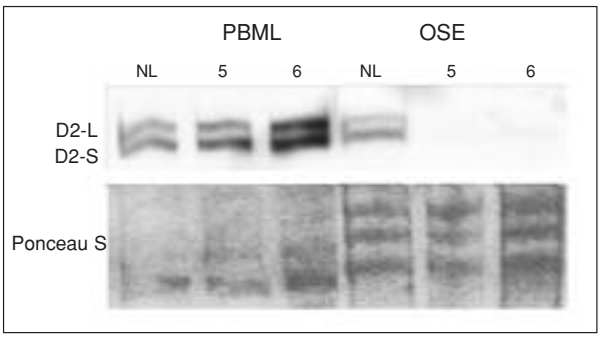


**Figure 2.** PBML and OSE protein lysated immunoblotted for FANCD2 protein. Bottom, Ponceau S stain for total protein. While PBML show normal levels of FANCD2, protein expression is reduced in endometriotic ovarian cells.

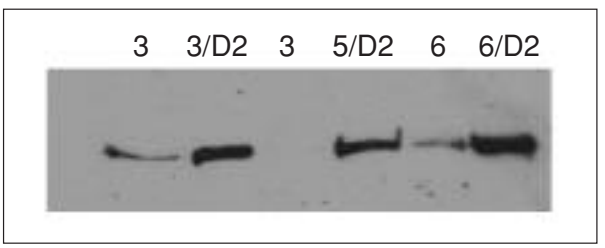


**Figure 3.** Restoration of FANCD2 expression and function (ubiquitination) after retroviral transuction of normal FANCD2 cDNA.

cells that were resistant to alkylating agents in the chromosomal breakage test. Two samples selected for further study, #5 and #6, showed markedly reduced levels of FANCD2-L and FANCD2-S protein isoforms, compared to normal control (Figures 1 and 2). In contrast, FANCD2 protein was readily detectable in PHA-stimulated peripheral blood lymphocytes from the same subjects (Figure 2).

To confirm that FANCD2 deficiency played a role in the genetic instability of #3, #5 and #6 cells, these cells were transformed with SV40 large T antigen, and then transduced with pMMP retrovirus containing the FANCD2 cDNA. After transduction of the normal FANCD2 cDNA, FANCD2 levels were restored, and the cells responded normally to exposure to the cross-linking agent as demonstrated by increased levels of FANCD2-L after treatment with MMC (Figure 3). Also, FANCD2 transduction of #5 and #6 cell lines significantly reduced the fraction of MMC-exposed cells bearing radial forms, from 55% to 28% (#5) and 44% to 20% (#6).

**No genetic loss of FANCD2 in epithelial cells with low FANCD2 expression**

Comparative genomic hybridization analysis on whole-genome oligonucleotide arrays was performed on the 2 samples (#5 and #6) by the method of Selzer et al. (15). In both samples, the FANCD2 gene locus was intact, with no gain or loss of 3p25.3 sequences at the array CGH resolution that was tested (6 kb median probe spacing, or twelve probes for the ~75 kb FANCD2 gene).

## Discussion

Genomic instability is a hallmark of cancer cells. However, some benign tumors (e.g., ovarian fibromas, uterine leiomyomas) and conditions do show evidence of nonrandom chromosomal changes (16). Endometriosis too demonstrates somatically acquired genetic alterations similar to those found in cancer, leading to clonal expansion of genetically abnormal cells (2,17). In addition, endometriotic cysts are monoclonal and characterized by the loss of heterozygosity in 75% of the cases associated with ovarian adenocarcinoma, and even in 28% of cases without accompanying carcinoma (18). Comparative genomic hybridization and LOH studies have revealed loss of DNA copy number changes on 1p, 5 q, 6q, 11g, 22q, and X, and gain on 6p and 17q . Fluorescent in situ hybridization (FISH) analyses confirmed that gain of 17q includes amplification of the proto-oncogene HER-2/neu in edometriotic foci, whereas endometriotic tissue adjacent to ovarian cancer frequently shows chromosomal imbalances of 9p, 11q, and 22q (19). Genetic aberrations, such as PTEN gene mutations, have been recognized in adjacent ovarian cancer and endometriotic lesions suggesting a possible malignant genetic transition spectrum (2,20). Endometriosis-associated ovarian cancers show higher expression of p53, c-erB-2 oncoproteins, and VEGF and reduced expression of estrogen and progesterone receptors than similar ovarian endometrioid cancer without associated endometriosis suggesting different pathways and cancer subtypes (2,21). Overexpression of p53 and bcl-2 proteins involved in apoptosis and metalloproteinase-9 (MMP-9) involved in basement membrane dissolution has been reported in cancers associated with endometriosis when compared to benign controls (20,21).

Epigenetic changes resulting in an abnormal expression of tumor suppression gene PTEN and DNA mismatch repair gene hMLH1 were described in 8.6% of advanced stage endometriosis (17).

However to this date none of the changes describes has been defined to predispose the endometriotic foci to malignant transformation.

Studies on the pathogenesis of rare human chromosomal breakage syndromes, such as Fanconi anemia, have helped define the molecular basis of the linkage of defective DNA damage responses and cancer risk. Four lines of evidence link FA pathway to ovarian cancer. (reviewed in 4). First, BRCA2 is an FA gene, identical to FANCD1. Second, FANCF silencing by methylation has been described in primary ovarian tumors. Finally, FANCD2 deficient mice tend to develop epithelial ovarian tumors. Finally, we have described FANCD2 protein reduction in OSEs from women an increased risk for ovarian cancer who are BRCA negative. Based on this evidence and known association of endometriosis and ovarian cancer, we set to investigate the contribution of FA pathway to endometriosis.

Using the screening tool of MMC/DEB-induced chromosomal breakage (11), we examined genomic instability in 8 populations of cells derived from patients with endometriosis. Our results demonstrated genetic instability in response to MMC challenge in 3 of the eight endometriosis samples. The finding is accompanied by reduced total FANCD2 levels but maintained FANCD2 capacity for monoubiquitination (a function that depends on all 8 FA core proteins).

Looking for the underlying cause of reduced FANCD2 protein expression we investigated the loss of heterozygosity at FANCD2 locus in these 3 samples. Although FANCD2 maps to 3p25.3, a chromosome region frequently lost in both sporadic human ovarian carcinomas and some cases of familial ovarian cancer (22), in this series we found no evidence of genetic loss in the epithelial cells with low FANCD2 levels ruling out a hemizygous loss of FANCD2 gene as a cause of FANCD2 protein reduction.

Our results implicate FANCD2 deregulation in endometriosis and show that this abnormality may be limited to OSE cells, as FANCD2 protein levels in peripheral blood lymphocytes from the same patients were not reduced, when compared to normal controls.

We have ruled out homozygous loss of FANCD2 as a cause of reduced FANMCD2 levels but we have not yet discovered a clear genetic or epigenetic mechanism for suppression of FANCD2 gene expression in these cells.

Although the data presented here is derived from a small series, there is clear indication after using cross-linking agent sensitivity testing that there is a high prevalence of genetically unstable epithelial cells in some women with endometriosis. As all three patients with unstable endometriotic cell populations had advanced endometriosis, these results may indicate that genomic instability is associated with loss of cell control and proliferation and distant spread.

To demonstrate conclusively identify that increased chromosomal breakage and low FANCD2 level predispose some women with endometriosis to cancer, one would need to follow the lesions prospectively. While this would will address a much needed insight into the pathogenesis of the disease, the effective screening would still require analysis of ovarian cells with MMC testing and FANCD2 immunoblots. While this approach may not be practical for all patients, it may be of value for young women with endometriosis who want to evaluate their risk of cancer while preserving fertility.

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