Ovarian Carcinoma In Situ With Germline BRCA1 Mutation and Loss of Heterozygosity at BRCA1 and TP53


Background: The two-hit hypothesis for the genetics of cancer predicts that cancer can develop when the wild-type allelle of a tumor suppressor gene is lost in an individual with a germline mutation in that gene. Neither loss of heterozygosity (LOH) for BRCA1 nor mutations of the TP53 (also known as p53) gene have been documented prior to invasion in ovarian cancers arising in women with germline BRCA1 mutations. Such documentation is difficult because lesions are rarely identified in ovarian epithelium. We, therefore, looked for LOH at microsatellite polymorphisms linked to the BRCA1 and TP53 tumor suppressor loci in an incidental carcinoma in situ of the ovary removed prophylactically from a woman with a germline BRCA1 mutation. Methods: By use of laser-capture microdissection, we obtained pure populations of atypical ovarian epithelial cells and normal stromal cells. DNA was extracted, amplified with primers flanking polymorphic microsatellites linked to BRCA1 (D17S855 and D17S579) and TP53 (TP53 and D17S786), and analyzed for LOH at these microsatellites. We also tested for p53 expression in the abnormal epithelium by immunohistochemistry. Results: Both of the markers linked to TP53 showed LOH, as did an intragenic BRCA1-linked marker (D17S855). The other microsatellite marker for BRCA1 was uninformative. Immunohistochemical staining with an antibody to p53 showed strong immunoreactivity confined to the atypical epithelium. Conclusions: BRCA1, as well as TP53, can undergo LOH prior to stromal invasion in BRCA1-associated ovarian cancer. Strong immunoreactivity for p53 suggests the presence of mutated p53 in these cells as well. These findings suggest that loss of function of these two tumor suppressor genes occurs early in ovarian carcinogenesis in BRCA1 mutation carriers. [J Natl Cancer Inst 2000;92:1088–91]

Women who inherit germline mutations in the BRCA1 tumor suppressor gene have increased risk for ovarian cancer (1). Consequently, these women often choose to have their ovaries removed prophylactically in an attempt to prevent the development of this disease. An incidental ovarian serous carcinoma in situ was discovered following hysterectomy and bilateral salpingo-oophorectomy for uterine bleeding in a woman with a germline mutation in the BRCA1 gene. We decided to analyze this rare case for loss of heterozygosity (LOH) in the BRCA1 and TP53 (also known as p53) genes at selected loci and for expression of p53 protein by immunohistochemistry.

Subjects and Methods

Specimens. Peripheral blood samples were obtained from the case subject, a female maternal first cousin, and a healthy, unrelated adult female (2). Leukocytes were isolated by use of the Puregene Kit (Gentra Systems, Minneapolis, MN) (3). In this procedure, whole blood is made hypotonic to lyse red blood cells. White blood cells are collected by centrifugation at 25 °C for 30 minutes at 1000 g, extracted with detergent, and sequentially digested with ribonuclease and proteinase K. Residual protein is removed by high salt precipitation. DNA was collected by precipitation with isopropanol and solubilized in 10 mM Tris (pH 8), containing 1 mM EDTA. Hematoxylin–eosin-stained pathology slides and pathology reports were obtained from the treating hospital for the case subject. Paraffin-embedded blocks of the ovaries were also obtained. Written informed consent was obtained from all subjects, and all specimens and medical records were obtained with protocols approved by the Institutional Review Boards of Roswell Park Cancer Institute (NY), Stanford University (CA), and the University of Cincinnati (OH). The Ponder Laboratory at Cambridge University (U.K.) is approved for germline genetic testing.

Mutation analysis. BRCA1 was amplified from the DNA of peripheral white blood cells in 40 fragments and was screened for mutations by use of combined single-strand conformation polymorphism (SSCP) and heteroduplex analysis (HA). Mutations in samples showing alterations by SSCP or HA were identified by sequencing an independent DNA amplification product (4).

Microdissection of tissue. Microdissection of abnormal epithelial cells and stromal cells from fixed, paraffin-embedded sections of ovary was done by use of an Arcturus PixCell II Laser Capture microdissecting microscope (Arcturus Engineering, Inc., Mountain View, CA). This system utilizes a transparent thermoplastic film applied to the surface of the tissue section on standard histopathology slides. The ovarian epithelial or stromal cells to be microdissected were identified and targeted through a microscope, and a narrow (~15 μM) carbon dioxide laser-beam pulse specifically activated the film above these cells. The resulting strong focal adhesion allowed selective procurement of only the targeted cells.

p53 immunohistochemistry. Tissue sections of 4 μm were cut, deparaffinized, and stained with a monoclonal antibody to p53 (clone DO-1, Oncogene Research, Cambridge, MA) at a dilution of 1:300. Sections were subjected to heat-induced antigen retrieval overnight at 75 °C in citrate buffer prior to addition of the primary antibody. All staining was carried out by use of the avidin–biotin method on a Ventana 320 Automated System (Ventana Medical System, Tucson, AZ). Diaminobenzidine was used as the chromogen. Negative controls consisted of substituting an isotypic mouse immunoglobulin G (IgG) (MOPC-21, IgG, κ; Sigma Immunochromicals, St. Louis, MO) for the primary antibody. The positive control consisted of a breast carcinoma from the surgical pathology files of the University of Cincinnati that was known from previous analysis to overexpress p53 protein.

LOH analysis. Two microsatellite markers linked to BRCA1 (D17S786 and D17S579) and to TP53 (TP53 and D17S555) were analyzed for LOH. DNA from microdissected tissue was extracted in 50 μL of solution containing 0.04% protease K, 0.5% Tween, 50 mM Tris (pH 8.5), and 1 mM EDTA (pH 8.0) at 37 °C for 24 hours followed by heat inactivation at 95 °C for 5 minutes. One microliter of the solution containing DNA was added to 13-μL polyme- rase chain reaction (PCR) mixtures described as follows: For D17S786, TP53, and D17S579, these contained 2.5 mM of each deoxynucleoside triphosphate (dNTP) (Panvera, Madison, WI), 7 μM of each primer, 1× ExTaq buffer, and 1.25 U of TaKaRa Ex-Taq DNA polymerase (Panvera). For D17S555, the amplification mixture contained 10 μL each of dNTP, 7 μM of each primer, 1.25 U of MasterTaq, 1× TaqMaster buffer, and 1× TaqMaster PCR enhancer (Eppendorf, Westbury, NY). Primers with the follow-
ing sequences were synthesized by the University of Cincinnati Core Biopolymer Facility: D17S856—forward 5′-TACAGGATAGTACCCGAG-3′ and reverse 5′-GGATTTGGGCTCTTTTGTAAA-3′; D17S855—forward 5′-ACACAGACTGTCCTAC-TGCC-3′ and reverse 5′-GGATGGGCCTTT-TAGAAAGTGG-3′; D17S579—forward 5′-AGGGATACTATTCAGCCCGAGGTG-3′ and reverse 5′-ACTGCCACTCCTG-CCCATC-3′. (Note: TP53 is used to designate this specific microsatellite polymorphism as well as the gene, TP53.)

Fluorescent labels were incorporated at the 5′ end of the forward primers. Touchdown PCR conditions employed were as follows: denaturation at 95°C for 5 minutes; 20 cycles of denaturation at 95°C for 40 seconds, annealing temperature plus 10°C for 40 seconds (decreased by 0.5°C per cycle), and extension at 72°C for 1.5 minutes; 10 cycles of denaturation at 95°C for 40 seconds, annealing temperature for 40 seconds, and extension at 72°C for 1.5 minutes; and a final extension of 72°C for 10 minutes.

The annealing temperatures were 58°C, 55°C, 58°C, and 50°C for D17S856, D17S855, TP53, and D17S579, respectively. PCR products were detected, and microsatellite allele sizes were determined on an ABI 377 sequencing instrument (Perkin-Elmer Applied Biosystems, Foster City, CA) with the use of 1 µL of amplified product. Visual inspection of electropherograms and Genotyper 2000 software (Perkin-Elmer Applied Biosystems) were used to compare the relative intensities of the two alleles and to determine LOH by use of criteria recommended by the manufacturer. These criteria determine the ratio of the peak heights on the electropherogram corresponding to the tumor and normal alleles; if this ratio is less than 0.67 or greater than 1.35, then LOH is established.

RESULTS

Case report. A 59-year-old woman underwent a hysterectomy and bilateral salpingo-oophorectomy because of persistent uterine bleeding while on tamoxifen therapy. She had begun taking tamoxifen 1 year earlier following wide local excision of a moderately differentiated, infiltrating ductal carcinoma of the left breast. Despite a benign endometrial biopsy 1 month previously, the patient elected to undergo a hysterectomy because of concerns about tamoxifen-induced endometrial cancer. In addition to her personal history of breast cancer, the patient reported that her mother had died of breast cancer, her maternal grandmother had died of ovarian cancer, two of her maternal aunts had died of breast and ovarian cancers, respectively, and a female maternal first cousin had developed breast cancer. Because of her strong family history of breast and ovarian cancers and her personal history of breast cancer, she enrolled in the Gilda Radner Familial Ovarian Cancer Registry, and slides and tissue blocks of her ovaries were obtained by the Registry for review. Analysis of DNA extracted from the patient’s peripheral blood leukocytes revealed a germline mutation of BRCA1 (525insA) in exon 7, codon 136. This mutation causes a shift in the codon reading frame and generation of a premature stop at codon 141, predicting synthesis of a truncated protein. The mutation also was found in DNA from a maternal first cousin who reported developing breast cancer at age 42 years.

Pathology. The original pathology report describes the ovaries as grossly unremarkable, yellow–tan, bosselated structures appropriate for the patient’s age. The greatest measurements from the glass slides are 1.8 cm and 2.3 cm for the right and left ovaries, respectively. Histologically, normal, flat to cuboidal surface epithelium is seen focally on both ovaries, but the left ovary exhibits a small focus of markedly atypical epithelium on the ovarian surface that extends focally into stromal invaginations of the surface epithelium (Fig. 1, A and B). The cells comprising this epithelium are enlarged, stratified, and exhibit loss of polarity (Fig. 1, C). The cytologic and nuclear features are those of a high-grade carcinoma. These features include marked nuclear pleomorphism, coarse chromatin with irregular chromatin distribution, irregular...

Fig. 1. Carcinoma in situ of the ovarian surface epithelium. Panel A: low-power (×6.25) magnification showing normal ovarian architecture. The bracket designates the region with neoplastic surface epithelium. Size bar = 700 µm. Panel B: higher magnification (×50) showing epithelium confined to the surface and extending into pre-existing epithelial invaginations, without stromal reaction. Size bar = 85 µm. Panels C–E: high-magnification (×400) views showing nuclear stratification, pleomorphism, and high mitotic activity. Size bar = 12 µm. Panel F: p53 immunohistochemical stain with immunoreactivity restricted to nuclei of abnormal cells (×400). Size bar = 12 µm.
macronucleoli, and frequent mitoses (Fig. 1, C and D). Features of stromal invasion, such as an irregular epithelial/stromal interface, single cells or small groups of cells within stroma, and stromal reaction, are not present. The interface between malignant and normal surface epithelium is present in several areas and exhibits an abrupt boundary, without an area of transition (Fig. 1, E). Strong nuclear immunoreactivity for p53, the protein product of TP53, is present and is confined to the abnormal cells (Fig. 1, F).

**LOH analysis at the BRCA1 and TP53 loci.** Neoplastic epithelia before and after microdissection from adjacent stroma are shown in Fig. 2. DNA extracted from stromal cells showed that the patient was informative for microsatellite polymorphisms linked to BRCA1 (D17S855) and TP53 (D17S786 and TP53) (Fig. 3, A–C, upper tracings). Two distinct peaks are seen corresponding to the two alleles. DNA that was extracted from the microdissected abnormal epithelial cells showed almost complete loss of one of the two alleles for these three markers, consistent with LOH (Fig. 3, A–C, lower tracings). The BRCA1-linked microsatellite D17S579 was not informative (Fig. 3, D).

**DISCUSSION**

Knudson’s two-hit hypothesis predicts the loss of the normal wild-type allele in tumors of patients who carry a germline mutation of a tumor suppressor gene (5). Reports that invasive cancers in women with germline BRCA1 mutations exhibit LOH at the BRCA1 locus (6–10) suggest that BRCA1 behaves as a classical tumor suppressor gene. Because a germline mutation in a tumor suppressor gene confers the earliest of genetic hits in a cell that subsequently generates a malignancy, it is plausible that loss of the wild-type allele is an early event, possibly occurring even prior to the acquisition of the capacity for invasion. To our knowledge, such a loss has not been documented in preinvasive lesions of either the breast or the ovary that occur in patients with germline BRCA1 mutations. Preinvasive LOH is particularly difficult to establish in ovarian cancers, since preinvasive carcinomas arising in the ovarian epithelium are rarely seen. By the time symptoms appear, the invasive tumor usually has completely obliterated all residual epithelium and any precursors from which it may have arisen. On rare occasions, early cancers not grossly identifiable have been found incidentally in ovaries removed for benign disease or on re-examination of ovaries previously removed from women for benign disease who later present with recurrent cancer consistent with an origin in the ovary (11). However, these cancers are almost always already invasive. The presence of a rare carcinoma in situ of the ovarian surface epithelium in a carrier of a germline BRCA1 mutation provided an opportunity to test for LOH in neoplastic epithelium obtained by laser capture microdissection. Molecular analysis of DNA from the microdissected abnormal epithelium showed LOH at the BRCA1 locus, confirming the prediction that LOH occurs prior to invasion.

Similarly, there have been difficulties...
in determining the timing of TP53 alterations in ovarian cancer. Attempts to classify a TP53 mutation as an “early” or “late” event in ovarian carcinogenesis based on the relative proportion of patients showing mutations in early- or late-stage ovarian cancers have generated conflicting opinions (12–21). However, such classification cannot address the question of whether or not TP53 is mutated prior to invasion. The abnormal ovarian epithelial cells in the patient on whom we report showed both LOH near the TP53 locus and strong immunoreactivity for p53. Normally, cellular p53 has a short half-life, contributing to low steady-state levels of p53 that are not detected by immunohistochemistry. However, TP53 missense mutations alter the structure of p53, resulting in a longer half-life, greater stability, and detection by immunohistochemistry (22). Thus, there is strong evidence for both mutation of TP53 and LOH of the remaining allele. Although TP53 mutations do not appear necessary for ovarian carcinogenesis in women with germline BRCA1 mutations (22,23,24), they appear substantially more often in BRCA1-related ovarian cancers than in sporadic ovarian cancers (23,24).

In summary, we have identified a carcinoma in situ in an ovary removed prophylactically from a woman with a germline BRCA1 mutation. Analysis of DNA from microdissected abnormal cells showed LOH at loci linked to both the BRCA1 and TP53 genes, as well as immunohistochemical evidence for mutation of the TP53 gene. This is the first molecular analysis of this rarely seen lesion and provides insight into the development of carcinoma in these patients.

REFERENCES


NOTES

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