Molecular Evidence for Multifocal Papillary Serous Carcinoma of the Peritoneum in Patients With Germline BRCA1 Mutations

John O. Schorge, Michael G. Muto, William R. Welch, Christina A. Bandera, Stephen C. Rubin, Debra A. Bell, Ross S. Berkowitz, Samuel C. Mok*

Background: Papillary serous carcinoma of the peritoneum (PSCP) diffusely involves peritoneal surfaces, while it spares or only superficially involves the ovaries. PSCP is histologically indistinguishable from serous epithelial ovarian carcinoma, and it may develop years after oophorectomy. The molecular pathogenesis of PSCP remains unresolved, although preliminary data suggest a multifocal origin in some cases. Patients with germline BRCA1 mutations may develop PSCP in addition to breast and ovarian carcinomas. The purpose of this study was to utilize the androgen receptor (AR) gene locus to test the hypothesis that some cases of PSCP have a multifocal origin and to determine if patients with germline BRCA1 mutations develop multifocal PSCP. Methods: Specimens of normal and tumor tissues from 22 women with PSCP were obtained, and DNA was extracted. The AR gene locus was evaluated for patterns of loss of heterozygosity (LOH) and X-chromosome inactivation. The methylation-sensitive Hpa II restriction enzyme was used to differentiate the active and inactive X chromosomes. Germline BRCA1 mutation status of the patients was determined previously. Results: Genetic analysis of tumor specimens indicated that five (23%) of 22 case subjects had patterns of selective LOH at the AR locus, consistent with multifocal, polyclonal disease origin. Two patients with selective LOH also had alternating X-chromosome inactivation patterns. Patients with germline BRCA1 mutations were more likely to have evidence of multifocal disease (two-sided Fisher’s exact test, $P = .01$). Conclusions: Our results show that PSCP has a multifocal origin in at least some cases. Furthermore, patients with germline BRCA1 mutations are more likely to develop multifocal PSCP than are patients without BRCA1 mutations. [J Natl Cancer Inst 1998;90: 841–5]

Papillary serous carcinoma of the peritoneum (PSCP) has an estimated annual incidence of 1000 new cases each year in the United States (1–6). PSCP diffusely involves peritoneal surfaces, while it spares or only superficially involves the ovaries (7). PSCP is histologically indistinguishable from papillary serous ovarian carcinoma, yet it may develop in a woman years after having an oophorectomy for benign disease or after having prophylactic oophorectomy for a family history of ovarian cancer (8–10). The staging, treatment, and prognosis of PSCP are similar to those of epithelial ovarian cancer (6,7).

Allelic losses at chromosomal loci are commonly found in advanced epithelial ovarian cancer (11–14). Analysis of the pattern of allelic loss, i.e., loss of heterozygosity (LOH), allows detection of chromosomal deletions in one of two alleles at a specific locus. A pattern of identical LOH in multiple tumor sites would be expected in a monoclonal, unifocal tumor. Identical patterns of LOH, p53 gene mutation, and X-chromosome inactivation have been reported in sporadic epithelial ovarian carcinoma (11,12,14–16). Although PSCP resembles primary ovarian carcinoma both histologically and clinically, its pathogenesis remains unresolved. A different pattern of LOH at multiple tumor sites within the same patient has been reported in three of six patients with PSCP (17). In addition, the findings of different patterns of p53 gene mutation and overexpression at various tumor sites within the same patient have suggested a multifocal origin in some cases of PSCP (17,18). Since unifocal tumors could potentially acquire genetic alterations such as LOH and p53 gene mutations during the course of progression and metastasis, stronger evidence would support more convincingly the hypothesis that PSCP is a multifocal disease.

Germline BRCA1 mutations occur in PSCP at a frequency comparable to that of epithelial ovarian cancer. Although the penetrance is unknown, PSCP should be considered a malignancy expressed in the breast–ovarian cancer syndrome (19). Knowledge of the molecular pathogenesis of disease in patients with BRCA1 mutations who develop PSCP would be clinically useful in developing strategies aimed at the prevention, prophylactic intervention, and early detection of mullerian papillary serous carcinoma.

The androgen receptor (AR) gene appears to be involved in the development of borderline and epithelial ovarian carcinomas (20,21); however, to our knowledge, its involvement in the pathogenesis of PSCP has not been investigated previously. The AR gene is X-linked and

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highly polymorphic and is located at mapping region Xq12. Unlike other loci, e.g., hypoxanthine phosphoribosyltransferase gene (HPRT) and phosphoglycerate kinase (PGK), used for the analysis of X-chromosome inactivation, the AR gene has two Hpa II methylation sites within 60 base pairs of its 90% polymorphic (CAG)n repeat (21,22). Enzymatic digestion cleaves the unmethylated Hpa II sites in the active allele, enabling identification of the remaining inactive X chromosome. Hpa II digestion followed by polymerase chain reaction (PCR) amplification of the inactive X chromosome can be reliably used to assess clonality in fresh and archival tissue specimens (23,24).

X-chromosome inactivation analysis has proved to be a powerful tool in the study of human cancer (11,14,23–25). The process of inactivation of the X chromosome in somatic cells is random and occurs at an early stage of embryogenesis. Once the specific parental X chromosome has been inactivated in a somatic cell, the same pattern will be transmitted to all daughter cells. If the multiple PSCP tumors in an individual patient are independent in origin, different parental X chromosomes may be inactivated.

To test the hypothesis that some cases of PSCP have a multifocal origin and to determine if patients with germline BRCA1 mutations develop multifocal PSCP, we analyzed allelic loss and X-chromosome inactivation patterns at the AR locus.

Patients and Methods
Diagnosis and Staging
All 22 case patients included in this study were diagnosed as having disseminated PSCP in the presence of normal-sized ovaries that either were entirely uninvolved or had small capsular implants with little or no stromal involvement. Each histopathologic diagnosis as well as assignment of grade was confirmed by a gynecologic pathologist (W. R. Welch or D. A. Bell). All tumors were surgically staged according to criteria of the International Federation of Gynecology and Obstetrics (26). Institutional review board approval was obtained from Brigham and Women’s Hospital and Massachusetts General Hospital to retrieve survival data and patient status as of November 1, 1997, from hospital charts, computerized patient records, and correspondence with health care providers.

DNA Extraction
DNA was extracted from both formalin-fixed, paraffin-embedded archival material and fresh surgical specimens collected under a protocol approved by the Human Subjects Committee of Brigham and Women’s Hospital. DNA specimens from eight cases were obtained from archival material (case subjects 6, 26–30, 32, and 33), 12 from fresh surgical specimens (case subjects 16, 25, 34–42, and 44), and two from combining both sources (case subjects 22 and 31). In blocks of tissue exhibiting focal tumor involvement, areas with at least 70% tumor cells were microdissected prior to DNA extraction. In each case, normal control DNA was extracted from either uninvolved round ligament, fallopian tube, or bowel serosa. DNA was extracted as described previously (16,27).

X-Chromosome Inactivation
X-chromosome inactivation was as described previously (21). Briefly, 2 μg of DNA from tumor or normal tissue specimens was digested overnight at 37 °C in a 20-μL reaction mixture containing the appropriate buffer and 20 U of Hpa II restriction endonuclease (Boehringer Mannheim Corp., Indianapolis, IN). Control reactions consisting of normal or tumor DNA samples incubated in the digestion buffer without Hpa II were also performed. After ethanol precipitation and resuspension in 10 μL of H2O, 1 μL of DNA from each reaction mixture was used as a template for PCR amplification with the use of commercially available oligonucleotide primers that flank the polymorphic CAG repeat region in exon 1 of the AR gene (Genosys Biotechnologies, Inc., The Woodlands, TX).

Cases with two allelic bands of equal intensity in normal tissue and at least two tumor sites with a heterozygous pattern were considered informative. We excluded cases with only one available tumor site, homozygous cases, and cases having an unequal, or skewed (15,23–25,28), band intensity pattern in normal control tissue. A reproducible decrease of more than 80% in the band intensity of a single AR allele following Hpa II digestion was scored as clonal origin by visual examination conducted by two investigators working independently.

Loss of Heterozygosity
LOH was studied by PCR amplification of the (CAG)n polymorphism within the AR gene as described previously (21). One microliter of sense AR primer was end-labeled with the use of 1 μL of [γ-32P]adenosine triphosphate (ICN, Irvine, CA), 0.5 μL of T4 polynucleotide kinase (Boehringer Mannheim Corp.), and 0.5 μL of kinase buffer (Boehringer Mannheim Corp.). The reaction mixture was then diluted with distilled H2O to a total volume of 5 μL and incubated at 37 °C for 30 minutes. It was then diluted into a final volume of 320 μL of a primer–PCR mixture containing 40 μL 10X PCR buffer (0.1 M Tris–HCl [pH 8.3] and 0.5 M KCl), 40–60 μL 4 mM MgCl2, 20 μL 1.25 mM deoxynucleotide triphosphate mixture, 2 μL (10 U) Taq polymerase (Perkin-Elmer, Branchburg, NJ), and 1 μL antisense AR primer. Four microliters of this PCR mixture was mixed with 1 μL of 50 ng/μL genomic DNA. Amplification was carried out in a Perkin-Elmer thermocycler for 40 cycles (each for 1 minute at 94 °C, 1–2 minutes at 50–52 °C, and 1–2 minutes at 72 °C), following an initial denaturation at 94 °C for 10 minutes. The 5-μL PCR product was then mixed with 45 μL of buffer containing 95% formamide, 20 mM EDTA (pH 8.0), 0.05% bromophenol blue, and 0.05% xylene cyanole FF (all from Sigma Chemical Co., St. Louis, MO). Aliquots (3 μL) were loaded onto a preheated 6% polyacrylamide gel (ratio of acrylamide to bisacrylamide, 19:1) containing 7 M urea and 10% TBE buffer (i.e., Tris–boric acid–EDTA [pH 8.0]) and subjected to electrophoresis at 1700 V. The gel was placed on a sheet of 3MM paper (Whatman, Maidstone, U.K.), dried, and autoradiographed with X-Omat AR film (Eastman Kodak, Rochester, NY). LOH was defined as a visible reduction of 50% or more in the band intensity of one of the tumor sample alleles when compared with the normal tissue control.

Direct Cycle Sequencing of PCR Product
Case 31 exhibited loss of one allele, with mobility shift of the other parental allele. The shifted band was cut from the gel and reamplified as described previously by use of the same primers and reaction conditions (16). The PCR product was purified by electrophoresis in a 1% agarose gel containing 5% TBE buffer and sequenced according to the method of Mork et al. (27) by use of a commercial DNA sequencing kit (Amersham Life Science Inc., Cleveland, OH). The sequence was confirmed by sequencing both sense and antisense complementary DNA strands; a 6% polyacrylamide gel containing 7 M urea and 10% TBE buffer was used for this analysis.

BRCA1 Single-Strand Conformation Polymorphism and DNA Sequencing
Of 22 patients with PSCP, 17 had been screened previously for the presence of germline BRCA1 mutations by use of single-strand conformation polymorphism analysis and direct DNA sequencing (19). These results were used in the current study.

Statistical Analysis
Differences between frequencies of molecular evidence for multifocality in patients with and without germline BRCA1 mutations were analyzed by use of Fisher’s exact test; reported P values resulted from use of two-sided tests.

Results
Twenty-two case patients with PSCP who were diagnosed and treated at either Brigham and Women’s Hospital or Massachusetts General Hospital during the period from December 1990 through August 1997 were studied. The clinicopathologic characteristics and of follow-up information on the patients are presented in Table 1. The median age of the patients at the time of surgical staging was 59 years (range, 35–80 years). Ninety-one percent of the patients were Caucasian, and 9% were African-American.

The pattern of allelic loss at multiple tumor sites is summarized schematically in Fig. 1. Tumor tissue specimens from 20 (91%) (cases 6, 16, 22, 25–29, 32–42, and
(CAG)_n polymorphism of the AR gene; 10 (50%) of the 20 patients exhibiting (CAG)_n heterozygosity had LOH of at least one tumor site (cases 6, 16, 22, 25, 26, 27, 31, 32, 33, 35, and 38) (Fig. 2). Four case subjects (cases 25, 27, 28, and 29) were each found to have a different pattern of allelic loss at various anatomic sites. Case 27 is shown in Fig. 2, and cases 25 and 28 are shown in Fig. 3. One patient (case subject 31) who was homozygous at the AR locus had a single tumor site with loss of one allele and mobility shift of the other allele (Fig. 2). Direct DNA sequencing of the band exhibiting altered mobility revealed the presence of a (CAG)2 deletion in the (CAG)_n repeat sequence of the AR gene.

Tumor specimen DNA from six of 20 heterozygous patients with PSCP was considered uninformative for X-chromosome inactivation analysis: Of these six patients, three (case subjects 33, 36, and 44) had only one available tumor site for analysis and three (case subjects 26, 29, and 41) had unequal allelic band intensity, or a skewed pattern, following Hpa II digestion of DNA from normal tissue. Of the 14 case patients with PSCP for whom DNA analyses were informative, two (case subjects 25 and 28) had an alternating pattern of X-chromosome inactivation and 12 (case subjects 6, 16, 22, 27, 32, 34, 35, 37–40, and 42) had an identical pattern of X-chromosome inactivation. Cases 25, 28, and 40 are shown in Fig. 3.

Tumor from all informative case patients showed the retention of one AR allele after digestion, which suggests a clonal origin of PSCP (Fig. 3). Retention of a faint band representing the other allele was also present in most instances, possibly a result of either contamination of the sample with normal tissue or incomplete Hpa II digestion. DNA prepared from normal control tissue specimens in 19 of 22 case subjects showed an equal reduction in band intensity in the two alleles following Hpa II digestion, reflecting the polyclonal nature of the control tissue; DNA specimens from three case subjects exhibited a skewed pattern and were considered uninformative.

Multiple specimens of fresh (n = 4) and archival (n = 4) tissues from different normal tissue sites in case subject 25 were available. Specimens from one or two normal tissue sites were available from case subjects 27, 28, 29, and 31. DNA specimens from these normal control tissues were tested to exclude the possibility of PCR artifacts. No evidence of clonal variation was observed at any of the control tissue sites.

Patients with germline BRCA1 mutations were more likely to exhibit molecular evidence of multifocal disease (P = .01). All three patients in the current study with known BRCA1 mutations (case subject 27, single-base-pair substitution at nucleotide 3719 in exon 11 resulting in a missense mutation; case subjects 28 and 31, 185delAG germline mutations) had evidence of multifocality (19). Of 14 patients without BRCA1 mutations, two

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Table 1. Clinicopathologic characteristics of and follow-up information on patients with papillary serous carcinoma of the peritoneum

*Grade 1 = well differentiated; grade 2 = moderately differentiated; grade 3 = poorly differentiated (1).
†FIGO = International Federation of Gynecology and Obstetrics. Stage IIIB = abdominal–peritoneal implants ≤2 cm in largest diameter; stage IIIC = abdominal–peritoneal implants >2 cm or retroperitoneal or inguinal lymph node metastases; stage IV = distant metastases (26).
‡A/NED = alive/no evidence of disease; AWDz = alive with disease.
Discussion

By analyzing the LOH and X-chromosome inactivation pattern at the AR locus, we have demonstrated a multifocal origin in five (23%) of 22 PSCP cases. By studying LOH on chromosomes 1, 3, 4, and 17 and the mutational pattern of the p53 gene, we have previously reported evidence for a multifocal origin in four patients with PSCP (17). Of the four patients identified in that study, three (case subjects 25, 27, and 28) have evidence of multifocality in the present study. The fourth patient (case subject 30) was homozygous at the AR locus and therefore uninformative. These findings definitively show that at least some cases of PSCP are multifocal in origin.

The question of tumor multifocality was not applicable or could not be evaluated in eight case subjects because there was a single tumor site, skewing, or a homozygous pattern at the AR locus. Possible reasons why we could not demonstrate multifocality in the other patients exhibiting AR locus heterozygosity include overgrowth of a dominant tumor clone, an insufficient number of tumor sites may have been tested with the same LOH and X-inactivation pattern by random chance, and some case subjects with PSCP may have tumors that are unifocal.

Patients with PSCP who had germline BRCA1 mutations were more likely to exhibit multifocal disease ($P = .01$) than patients without BRCA1 mutations. Among the patients with PSCP who had identical LOH and X-chromosome inactivation patterns, no BRCA1 mutations have been identified. If these preliminary findings are confirmed, familial predisposition due to BRCA1 mutation may prove to be associated with a multifocal pathogenesis in patients with PSCP. We speculate that predisposing germline mutations other than BRCA1 may be associated with other cases having evidence of multifocality in this study.

To our knowledge, this is the first report to demonstrate the involvement of the AR gene in the development of PSCP. We found a similar frequency of allelic loss at the AR locus in patients with PSCP (50%) as has been previously reported for patients with ovarian cancer (38%–44%) (20,21). These findings suggest that the AR gene may be involved in the pathogenesis of PSCP as well as in the pathogenesis of ovarian cancer. In ovarian cancer, a 1-centimorgan deletion unit on chromosome Xq12 has been identified (21). Further investigation to search for mutations and common deletion units may elucidate a potential role for the AR gene in the pathogenesis of PSCP.

All cases showed retention of an AR allele following endonuclease digestion, indicating a clonal origin for PSCP. Histopathologic as well as clinical evidence has established the potential of the female upper genital tract to develop independent primary neoplasia at multiple anatomic sites (29). We have shown evidence of clonal tumor origin from multiple peritoneal sites (e.g., polyclonality) in patients with PSCP, supporting the hypothesis that cells derived from the coelomic epithelium may independently undergo transformation (8).

In conclusion, our study provides evidence that at least some patients with PSCP have a multifocal disease origin and that patients with germline BRCA1 mutations are more likely to develop multifocal PSCP than patients without BRCA1 mutations. These findings suggest that some cases of PSCP have a distinct molecular pathogenesis from epithelial ovarian cancer. In addition, the increased likelihood of multifocality in patients with germline BRCA1 mutations has implications for clinical strategies aimed at the prevention, prophylactic intervention, and early detection of müllerian papillary serous carcinoma.

References


(2) Berek JS. Epithelial ovarian cancer. In: Berek


Notes

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