Advances in Brief

X Chromosome Inactivation and Microsatellite Instability in Early and Advanced Bilateral Ovarian Carcinomas

Tjoong-Won Park, Juan C. Felix, and Thomas C. Wright, Jr.

Abstract

Ovarian carcinoma can arise synchronously from multiple independent sites and metastasize widely. Therefore, it is frequently unclear whether the tumors represent two independent primaries or one primary and a metastasis. We have used X chromosome inactivation of the androgen receptor gene and microsatellite instability at four chromosomal loci to evaluate the clonal origin of 39 bilateral ovarian carcinomas. An identical monoclonal pattern was found bilaterally in all cases including 10 stage I bilateral ovarian carcinomas. Microsatellite alterations were identified in three cases, and in all three, identical alterations were present in tumor tissue from both ovaries. These results suggest that bilateral ovarian carcinomas evolve as unifocal neoplasias and that metastatic dissemination can occur early in the course of the disease.

Introduction

Epithelial ovarian carcinomas comprise 90% of all ovarian neoplasms and have the worst prognosis among malignancies of the female genital tract (1). Poor outcome is frequently attributed to the fact that the tumors are often widely disseminated throughout the pelvis and abdomen at the time of diagnosis. Although disseminated disease is usually due to spread along the peritoneal surface to adjacent and distant organs, there is also evidence to suggest that these tumors can arise synchronously from multiple sites (1–3). For example, bilaterality is found at diagnosis in 20–25% of early stage ovarian tumors that are confined to the ovaries and do not extend the ovarian capsule (stage I) (4). Moreover, tumors with an identical histological appearance can arise from the peritoneal surfaces of women who have normal ovaries or who have had oophorectomies for benign disease (1, 5). The frequency with which ovarian carcinoma develops synchronously from multiple independent sites is an important consideration in therapy, especially in women who want to maintain their fertility, and in the development of strategies for early detection and prevention.

In this study we examined the clonal composition of early (stage I) and advanced (stages II, III, and IV) bilateral ovarian carcinomas of different histological subtypes to determine whether they develop as multifocal or unifocal tumors. The first strategy was to analyze X chromosome inactivation by detecting methylation differences adjacent to a polymorphic region of the androgen receptor gene (6). This gene contains a hypervariable trinucleotide sequence in exon 1, which is heterozygous (i.e., informative) in approximately 90% of women (7). The second strategy was to analyze the tumors for random microsatellite mutations at four different chromosomal loci. Genomic instability of such microsatellites has been described in a variety of tumor types and can be used as a clonal marker (8–11).

Materials and Methods

Seventy-eight bilateral formalin-fixed, paraffin-embedded ovarian tumors and the corresponding normal tissues (i.e., corpus uteri and cervix uteri) from 39 patients with bilateral tumors were examined. The specimens were obtained from the pathology archives of Columbia Presbyterian Medical Center (New York, NY) and the University of Southern California (Los Angeles, CA). Thirteen cases were classified as stage I, 1 case as stage II, and 25 cases as stages III and IV.

Normal and neoplastic tissue were identified on hematoxylin and eosin-stained tissue sections. Two 4-5-μm sections were cut from the paraffin block and the neoplastic tissue microdissected away from contaminating normal tissue under an inverted microscope. The DNA was extracted and purified as described previously (12).

For the analysis of X chromosome inactivation, a 5–10-μl aliquot was digested with 20 units HpaII restriction endonuclease (New England Biolabs, Beverly, MA) and ethanol precipitated before PCR. The 50 μl PCR reaction contained PCR buffer [60 mM Tris-HCl, 25 mM (NH4)2SO4, 2.5 mM MgCl2 (pH 9.5)], 2.5 mM concentration each of dNTP (Pharmacia, Piscataway, NJ), 1 unit Taq polymerase (Perkin Elmer Cetus, Norwalk, CT), and 100 pmol concentration of each radioactively end-labeled and unlabeled primers. Primers were endlabeled with [α-32P]ATP, >4500 Ci/mmol (ICN, Costa Mesa, CA). The primer sequences for amplification of the androgen receptor gene were (5′-3′):-CGG AGG AGC TTT CCA GAA TC and TAC GAT GGC CTT GGG GAG AA Operon Technologies (Alameda, CA).

Twenty-seven PCR cycles were performed at 95°C for 30 s, 55°C for 45 s, and 72°C for 90 s, with a final extension at 72°C for 7 min. Six μl of PCR product were mixed with 3 μl of loading buffer and loaded onto a denaturing 6% urea polyacrylamide gel. Autoradiography was performed by using Kodak XAR films (Eastman Kodak Co., Rochester, NY).

Analysis of microsatellite instability was performed by PCR by using three dinucleotide repeat markers (D2S119, D10S197, and D13S317) and one trinucleotide repeat marker (androgen receptor gene). The dinucleotide primer pairs were obtained from Research Genetics (Huntsville, AL). The PCR reaction for the dinucleotide repeat markers contained PCR buffer [60 mM Tris-HCl, 25 mM (NH4)2SO4, 1.5 mM MgCl2 (pH 9)], 2.5 mM concentration each of dNTP (Pharmacia, Piscataway, NJ), 1 unit Taq polymerase (Perkin Elmer Cetus), and 100 pmol concentration of each of the radioactively end-labeled and unlabeled primers. Thirty-five cycles of PCR were performed at 94°C for 40 s, 55°C for 30 s, and 72°C for 90 s with a final extension at 72°C for 7 min. Separation and visualization of the amplification products were performed as described above.

Results

Thirty-nine bilateral ovarian carcinomas were examined for non-random X chromosome inactivation and microsatellite instability. Twenty-nine (74%) of the 39 cases were heterozygous with respect to the (CAG)n polymorphism of the androgen receptor gene. In all heterozygous cases the band intensity of both alleles was determined by densitometry (Mocha; Image Analysis Software, San Rafael, CA). Twenty-six heterozygous cases demonstrated a polyclonal pattern with two distinct bands of approximately equal intensity in the non-cancerous tissues (i.e., corpus uteri and cervix uteri) Densitometry of
these 26 cases indicated that the ratio between the two bands averaged 47:53 (range, 45:55–50:50). Therefore, these cases were classified as informative. In contrast, in three cases a skewed pattern of X chromosome inactivation was observed in the polyclonal control tissue. By densitometry, the ratio of the two bands in these three cases averaged 40:60. Therefore, these cases were not included in the statistical analysis. Ten (38%) of the 26 informative cases were classified as stage I, and 16 (62%) were classified as stage III or IV (Table 1). In all 26 informative cases, the tumors obtained from both ovaries demonstrated only a single allele after amplification, indicating non-random X chromosome inactivation and a monoclonal origin.

In each of the 26 pairs of informative bilateral ovarian cancers, a PCR product of an identical size was identified in both tumor sides, suggesting that the same X chromosome was inactivated in both tumors (Fig. 1). Statistical analysis of these results indicates that it is highly unlikely that identical X chromosomes would be inactivated bilaterally in all cases by chance ($\chi^2$ test, $P < 0.001$). This suggests that a substantial number of bilateral ovarian carcinomas have a unifocal origin. Similar results were obtained when only stage I bilateral ovarian carcinomas were analyzed ($n = 10$, Fisher's exact test, $P < 0.03$).

All 39 pairs of bilateral ovarian carcinomas were also analyzed for the presence of microsatellite mutations by using four different repeat markers. Microsatellite mutations were identified in one stage I (case 1) and two stage III bilateral ovarian carcinomas (cases 18 and 23) but not in the other 36 cases (Table 1). In these three cases novel or shifted...
bands of the same size were present bilaterally at loci D2S119 (case 1) D10S197 (case 18) and the androgen receptor gene (case 23) (Fig. 2). None of the examined ovarian carcinomas demonstrated alterations at locus D13S175.

Discussion

In this study we have utilized the human androgen receptor gene as a clonal marker to determine whether bilateral ovarian cancers represent two independent primary tumors or one primary and a metastasis. During this investigation we have confirmed that the human androgen receptor gene has several distinct advantages over other commonly used clonal markers (e.g., PGK gene, glucose-6-phosphate dehydrogenase isoenzymes) (13, 14). The androgen receptor PCR-based clonality assay allows retrospective screening of archival, formalin-fixed, paraffin-embedded tissues and does not require fresh or frozen tissue specimens. Another advantage of this assay is that the majority of women are heterozygous (i.e., informative) for the polymorphism of the androgen receptor gene. In our study 74% (29 of 39) of the women were heterozygous for this polymorphism. Although this percentage is somewhat less than observed in another study, which reported that 90% of cases were polymorphic, it is still higher than observed with many other clonal markers (14). A skewed pattern of X chromosome inactivation was identified in 8% (3 of 39) of the cases in this study. Because we felt that this might make it difficult to interpret the results, these cases were classified as “inconclusive” and dropped from the results (15). The number of cases with a skewed X chromosome inactivation pattern is similar to that reported by Vogelstein et al. (15, 16) but less than recently reported by others. One possible explanation for the low rate observed in this study is that in previous studies interpretation of the results has been complicated because each allele was visualized by two major and several minor bands after internal labeling with radioactive nucleotides (6). In this investigation the labeling protocol was modified such that one of the primers was radioactively end labeled. Therefore, each allele is represented by a single major band, which simplifies the interpretation of the results.

As expected, in all 26 informative cases the tumors were monoclonal, whereas the control nontumor tissues were polyclonal. More importantly, an identical X chromosome inactivation pattern was identified bilaterally in every informative case. None of the informative cases had a discordant X chromosome inactivation pattern, which would have indicated that the bilateral tumors had developed as two independent primaries. Although it is possible that some of the bilateral tumors may have developed independently and demonstrated identical patterns of X chromosome inactivation by chance, statistical analysis of our results strongly suggests that bilateral ovarian carcinomas frequently have a unifocal origin. This conclusion is also supported by the evaluation of microsatellite alterations. Microsatellite alterations appear to be early events in various sporadic tumors and can also act as a clonal marker (10, 11). In this series, three of the cases had identical microsatellite alterations bilaterally, indicating that the bilateral tumors were clonally related. Discordant microsatellite alterations were not detected in any of the cases.

Despite the fact that ovarian carcinomas are common extracolonic tumors in hereditary nonpolyposis colon carcinomas (HNPCC) families, only 3 of 36 (8%) cases demonstrated microsatellite mutations. The low prevalence of microsatellite alterations detected in this study is consistent with the results of Wooster et al. (9) and suggests that ubiquitous genomic instability involving multiple microsatellite loci does not play an important role in the pathogenesis of bilateral ovarian cancer. Such ubiquitous genomic instability frequently occurs in hereditary nonpolyposis colon carcinomas and also at a lower frequency in HNPCC-associated carcinomas, as well as in various sporadic carcinomas (9–11).

Previous cytogenetic and molecular genetic studies of ovarian carcinoma have been performed on predominately advanced (stages III and IV) serous adenocarcinomas (17–19). In contrast to previous studies, our study included ovarian carcinomas of different histological types (i.e., serous, mucinous, endometrioid, clear cell, and undifferentiated ovarian carcinomas) of different clinical stages and different histological grades. An identical pattern of X chromosome inactivation was identified bilaterally, irrespective of histological type or grade. Moreover, ten informative cases of bilateral, stage I ovarian carcinoma (i.e., confined to the ovaries bilaterally) were included in this study, and in all ten cases an identical pattern of X chromosome inactivation was identified bilaterally. In one case of stage I bilateral ovarian carcinoma, identical microsatellite alterations were observed on both sides (case 1). These findings suggest that metastatic dissemination is an early event in the pathogenesis of ovarian carcinomas and have important implications for ovarian cancer screening programs, as well as for developing rational treatment protocols for women with early stage disease.

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References

CLONAL ANALYSIS OF BILATERAL OVARIAN CANCERS


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