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Advances in Brief

Evidence for the Multifocal Origin of Bilateral and Advanced Human Serous Borderline Ovarian Tumors[1]

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Abstract

Borderline ovarian tumors (BOTs), or ovarian tumors of low malignant potential, represent a distinct category of epithelial ovarian neoplasms that have a clinically more favorable outcome than invasive epithelial ovarian cancer. Histologically, BOTs and invasive ovarian carcinomas both show cellular proliferation and pleomorphism, but unlike invasive ovarian carcinomas, BOTs lack stromal invasion. Although serous BOTs are frequently confined to a single ovary at the time of diagnosis, bilateral or extra-ovarian spread occurs in 30–40% of cases.

The purpose of this study is to determine whether bilateral or extra-ovarian serous borderline lesions are metastatic sites from the original tumor, or represent separate primary tumors.

DNA specimens from multiple tumor sites and normal tissue controls were obtained in eight women with bilateral or extra-ovarian serous borderline tumors. The pattern of loss of heterozygosity at the androgen receptor locus on the X chromosome was evaluated in the multiple tumor sites. In addition, the pattern of X-chromosome inactivation was determined using *Hpa*II restriction endonuclease digestion, followed by PCR amplification of the androgen receptor locus. Multifocality was determined when alternate patterns of X-chromosome inactivation occurred.

In two of the eight patients, the left and right ovarian tumor sites had different androgen receptor alleles inactivated, indicating that the bilateral tumors derived independently. In a third patient, the X inactivation pattern in the left ovarian tumor differed from the two peritoneal implants, suggesting that the implants were separate primary tumors, and not metastatic, from the left ovarian tumor. The remaining five patients had the same pattern of loss of heterozygosity and X inactivation in the tumor sites studied.

These results suggest that bilateral and advanced stage serous BOTs may be multifocal in origin. This result is in contrast to invasive epithelial ovarian cancer, which has been shown to be unifocal in origin.

Introduction

In 1961, the International Federation of Gynecology and Obstetrics recognized a distinct category of epithelial ovarian neoplasms that was intermediate between the benign and malignant subtypes (1). In contrast to invasive ovarian cancers, BOTs,[3] or ovarian tumors of low malignant potential, tend to occur in younger women, present at an earlier stage of disease, and have an excellent overall 5-year survival rate of 95% (2–10). Histologically, BOTs are characterized by cellular proliferation and pleomorphism but, unlike invasive ovarian cancers, they lack stromal invasion (11–13). Although the majority of serous borderline tumors are confined to a single ovary, approximately 30–40% will present as bilateral or advanced stage disease (3, 5, 6, 8, 9).

The pathogenesis of serous BOTs at multiple sites has been the topic of controversy for many years. Whether there is a single ovarian tumor that metastasizes or "seeds" the other ovary and peritoneum, or whether there is a "field defect" causing multiple primary tumors to occur simultaneously is unknown. In this study we used LOH and X-chromosome inactivation techniques which provided evidence for the latter theory, or the multifocal origin of serous BOTs.

X-chromosome inactivation techniques have been used to study focality in a number of human cancers (14–19). Because all somatic cells of females randomly inactivate one of their X chromosomes early in embryogenesis, the majority of human tissues, which are polyclonal, have an approximately equal distribution of inactivated maternal and paternal X alleles. Therefore, the stem cells for the germinal epithelium of the ovary and for the mesothelium of the peritoneal lining contain a random distribution of inactivated maternal and paternal X alleles. However, for a specific progenitor, all daughter cells will inherit the same inactivated X chromosome. This is the basis for using the inactivated X chromosome as a marker of clonality for tumors and of focality for multiple tumor sites.

In this study, we used the AR locus on chromosome Xq11–12 for analysis of the pattern of X-chromosome inactivation (20). In addition, we examined the pattern of LOH at the AR locus in multiple tumor sites, because LOH at the AR locus has been shown to occur in about 25% of serous borderline tumors (21).

Materials and Methods

Specimens from six patients with bilateral or advanced serous BOTs were obtained from pathology at the time of surgery under a protocol approved by the Human Subjects Committee of the Brigham and Women's Hospital. All histopathological diagnoses of serous BOTs were confirmed by a gynecological pathologist. Fresh tumor tissue was carefully dissected from surrounding normal tissue. Control tissue was obtained from uninvolved fallopian tube or round ligament. Both normal and tumor tissue specimens were incubated in digestion buffer (5 M NaCl, 1 M Tris-Cl, 0.5 M EDTA, 10% SDS, and 0.1 mg/ml proteinase K) overnight at 50°C, followed by a series of phenol: chloroform extractions interrupted by an Rnase digestion as described previously (14). The DNA was finally precipitated in cold ethanol with sodium acetate and resuspended in Tris-EDTA buffer, pH 7.5.

One microgram of DNA from each tumor and normal specimen were digested with 20 units of *Hpa*II restriction endonuclease (Boehringer Mannheim, Indianapolis, IN) in a total volume of 20 μl. Control reactions for each sample were incubated in the digestion buffer without endonuclease. After digestion overnight at 37°C, 1 microliter of DNA from each sample was used as a template for PCR amplification using a primer set flanking a polymorphic CAG repeat region in exon 1 of the AR gene (20).

The primer set for the microsatellite marker AR was obtained from Genosys (The Woodlands, TX): 5′ GCTGTGAAGGTTGCTGTTCCTCAT3′ and 5′ TCCAGAATCTGTTCCAGAGCGTGC3′. The forward primer was end-labeled with γ [^{32}P] ATP (ICN, Irvine, CA) using polynucleotide kinase (Boehringer Mannheim). The reaction mixture was then diluted into a final volume

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[3] The abbreviations used are: AR, androgen receptor; BOT, borderline ovarian tumor; LOH, loss of heterozygosity.

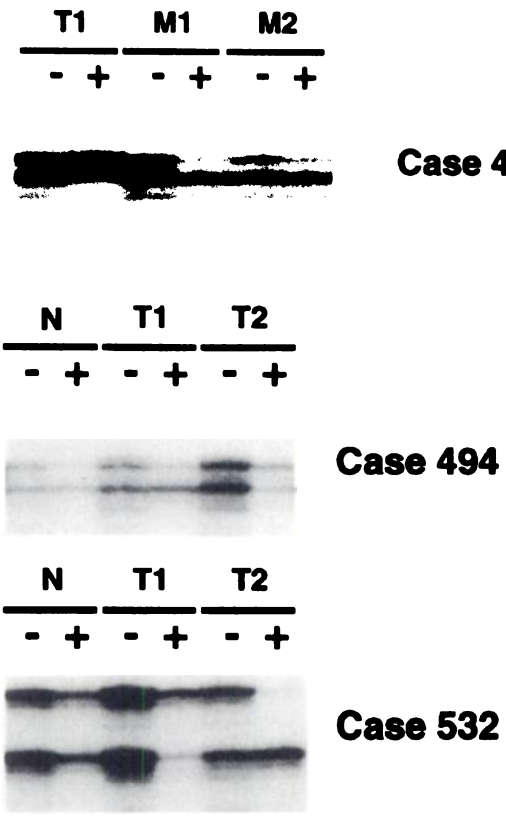

Fig. 1. Autoradiograph of one case (427) illustrating that ovarian tumor and peritoneal implants may arise independently, and two cases (494 and 532) illustrating that bilateral ovarian tumors may arise independently. N, normal tissue; T1, left ovary; T2, right ovary; M1 and M2, peritoneal implants. −, before *Hpa*II digestion; +, after *Hpa*II digestion.

of 360 μl of primer-PCR mixture containing 40 μl 10 × PCR buffer (0.1 M Tris-HCl, 0.5 M KCl, pH 8.3), 60 μl 4 mM MgCl2, 20 μl 1.25 mM dNTP, 2.0 μl (10 units) of *Taq* Polymerase (Perkins-Elmer Corp., Norwalk, CT), and 1 μl of each primer. Nine microliters of this PCR mixture was mixed with 1 μl of each DNA sample. Amplification was carried out using 35 cycles, with denaturation at 94°C for 30 s, annealing at 48°C for 30 s, and extension at 72°C for 30 s. PCR products were then diluted in 45 μl of loading buffer containing 95% formamide, 20 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol FF (Sigma Chemical Co., St. Louis, MO). The samples were heated to 99°C for 10 min, then placed on ice. Three microliters of this mixture were loaded onto a 6% acrylamide denaturing gel and electrophoresed at a constant 1700 V. The gel was transferred onto a 3 MM paper, dried, and autoradiographed with Kodak X-OMAT AR film.

The cases were considered informative if two bands, representing the maternal and paternal AR alleles, were seen after PCR amplification. Only informative cases were used for our studies. LOH was defined as a visible reduction of 50% or more in band intensity of one of the tumor sample alleles when compared with the normal tissue control. For the X-chromosome inactivation studies, AR allele intensities in the normal tissue samples were evaluated first. Cases with a skewed pattern of X-chromosome inactivation in the polyclonal control tissue were excluded. In the tumor tissue samples, a visible reduction of more than 80% in the band intensity of one of the two AR alleles compared with the remaining allele after *Hpa*II digestion indicated predominence of one AR allele and, therefore, clonal derivation of the tumor sample. Multifocality was presumed when alternate X alleles predominated after *Hpa*II digestion of different tumor sites within the same patient.

Results

LOH and X-chromosome inactivation of the AR locus was analyzed in 18 tumor samples from eight patients. In seven of eight patients, tumor samples were obtained from the right and left ovary. In two of the patients, tumor samples were obtained from both ovarian and extra-ovarian sites. The mean age of the six patients was 46 (range, 30–64). Recurrence information is limited because the majority of cases were diagnosed within the past 4 years. However, patient 395 was noted to present with papillary serous carcinoma of the peritoneum 4 years after optimal surgical debulking for Stage 2c serous BOT. No residual tumor was noted at the end of the initial surgery.

Given the random inactivation of maternal and paternal X alleles in cells early in embryogenesis, one would expect normal or control tissue to harbor an approximately equal distribution of cells with the maternal or paternal X inactivated. However, because a tumor is believed to be derived from the transformation of a single cell, the population of a single tumor should all have the same maternal or paternal X inactivated. In the control *Lanes* in Fig. 1, after digestion with *Hpa*II, approximately 50% of the signal was represented for each AR allele. In each of the *Hpa*II digested tumor *Lanes* in Fig. 1, the population of tumor cells all have the same X inactivated, strongly suggesting the clonal origin of individual borderline tumors.

We then used the X-inactivation technique to address the question of whether tumor from different sites in the same patient derive from the same parent cell. If tumors from the left and right ovaries were derived from the same transformed parent cell, the tumors would have the same X chromosome inactivated. If tumors from the two ovaries were derived from different parent cells, the tumors would have a 50% probability of having different inactivation patterns, and may have a different pattern of LOH. The results for Patient 532 and Patient 494 are shown in Fig. 2. In both cases the control tissue, after *Hpa*II digestion, showed an expected reduction in intensity equally in the two alleles reflecting the polyclonal nature of the control tissue. However, in both tumor cases, the left and right ovarian specimens showed alternate X alleles inactivated, strongly suggesting that these tumors were separate primary tumors.

In Patient 427, the two peritoneal tumor implants had the same LOH pattern and the same X-inactivation pattern after *Hpa*II digestion (Fig. 1). However, the left ovarian tumor specimen did not show LOH and had a different X allele inactivated than the two tumor implants. The peritoneal implants, therefore, were not derived from the same progenitor cell as the ovarian tumor. Histologically the tumor implants were also borderline serous tumor.

Patients 395, 466, 561, 595, and 607 all had the same pattern of

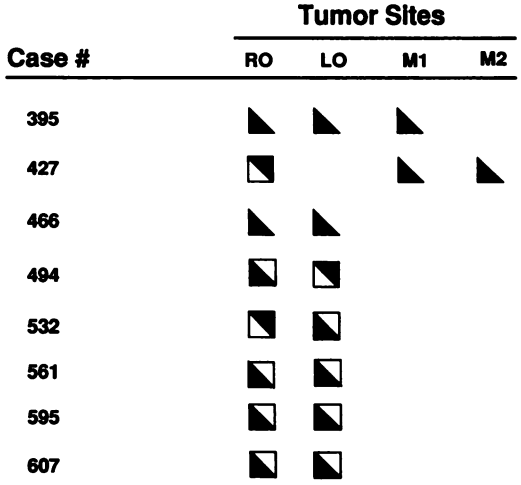

Fig. 2. Schematic illustration of LOH and X-chromosome inactivation patterns. *Shaded box* represents retained X-allele after *Hpa*II digestion. LO, left ovary; RO, right ovary; M1 and M2, peritoneal implants. ◥ heterozygous, inactivation of lower allele after *Hpa*II digestion; ◤ heterozygous, inactivation of upper allele after *Hpa*II digestion; ◣ LOH, inactivation of upper (inactive) allele after *Hpa*II digestion.

LOH and X-inactivation for the right and left tumor specimens. All of the results for the eight patients are shown in Fig. 2.

Discussion

This study provides evidence for the multifocal origin of serous BOTs. In three patients with bilateral or advanced serous BOTs, the tumor sites have different patterns of X-chromosome inactivation, suggesting that they derive independently from multiple sites. In the other five patients, the right and left tumors have the same pattern of X-inactivation. This result may suggest that the tumors from separate ovaries are derived from the same progenitor cell, or it may also suggest that, by chance, the same X-allele was inactivated despite being derived from different progenitor cells. Clearly bilateral or advanced stage serous borderline tumors can be multifocal.

This result is in contrast to invasive epithelial ovarian cancer and the majority of human tumors that derive from a single progenitor cell and then metastasize. Our laboratory and others have used X-inactivation techniques and allelic deletion previously to demonstrate that bilateral and advanced stage invasive epithelial ovarian cancer is unifocal in origin (14, 15, 18). Kupryjanczyk *et al.* (22) used p53 mutations to show that multiple sites from serous ovarian cancers, serous peritoneal cancers, and serous endometrial cancers also have a monoclonal origin. Other researchers have used similar techniques to demonstrate unifocality in other types of tumors in which multiple sites of tumor occur simultaneously, such as bladder cancer (16).

Our results are consistent with clinical observations of the behavior of BOTs. BOTs are known to recur many years after initial surgical debulking (2–5, 7, 23). The possibility that the recurrence may be a new primary tumor is certainly a strong possibility given these new findings of multifocality. In addition, peritoneal lesions associated with ovarian serous borderline tumors have a variable histological appearance, and can be classified as benign, noninvasive (borderline), or invasive (24, 25). We hypothesize that peritoneal implants may, in fact, be separate primary tumors. An invasive implant in a patient with a BOT may, in fact, be an independent early papillary serous carcinoma of the peritoneum. Our lab has shown previously that papillary serous carcinoma of the peritoneum may also be a multifocal process (26). Finally, two series have reported the existence of peritoneal borderline tumors without ovarian disease (27, 28). Because the cells of the peritoneal lining and ovarian surface epithelial cells all derive from coelomic epithelium, we postulate that these cells can independently undergo transformation to become separate borderline tumors.

In women with BOTs, what causes multiple primaries to arise simultaneously? Clinicians who previously suggested the multifocal origin of borderline tumors describe a "field defect" in which hormonal or environmental effects may be involved in the pathogenesis of this disease. However, epidemiological and other studies have not yet identified specific etiological agents. Other tumors that have been found to be multifocal are those that carry a genetic predisposition such as hereditary neurofibromas and colonic adenomas (17, 19). Thus far it is unclear whether BOTs have a familial component, and no germline mutations are known to be associated with BOTs.

Our study was limited by including only two cases of borderline tumor with peritoneal implants. Although bilateralism can occur in up to 52% of serous borderline cases, pelvic or peritoneal implants occur less frequently (9). Our results certainly justify an extended study of the focality of multiple peritoneal implants. We did not study mucinous or other histological types of BOTs. Mucinous borderline tumors infrequently present as bilateral or late stage disease (6). Whether mucinous or other histological subtypes of BOTs are unifocal or multifocal has not been established. In addition, the question of whether BOTs of any histological type can be transformed into invasive disease is also unknown.

In conclusion, our study demonstrates for the first time that bilateral and advanced stage serous BOTs may be multifocal in origin. Additional investigation into BOTs and their relation to invasive epithelial ovarian cancer is ongoing in our laboratory.

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