Second Primary or Recurrence? Comparative Patterns of p53 and K-ras Mutations Suggest that Serous Borderline Ovarian Tumors and Subsequent Serous Carcinomas Are Unrelated Tumors

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ABSTRACT

The role of serous borderline ovarian tumors (BOTs) in the pathogenesis of serous ovarian carcinomas is unclear. Some authors have compared mutations in serous BOTs to those in serous ovarian carcinomas, but the data on two common oncogenes, p53 and K-ras, remain inconclusive. To further clarify the relationship between the two tumors, we performed mutational analysis on tumors from a set of eight patients who first presented with advanced-stage serous BOTs and later developed grade I serous carcinomas. Epithelium from eight advanced-stage serous BOTs and subsequent grade I papillary serous carcinomas was microdissected and analyzed by single-strand conformational polymorphism-PCR for p53 and K-ras mutations. Bands with altered motility were analyzed by direct cycle sequencing. Seven of eight patients demonstrated different mutations in the secondary tumor compared with the primary tumor. For these patients, p53 mutations were identified in the BOTs that were absent from the carcinomas, suggesting a nonclonal origin for the carcinomas. These findings are consistent with the hypothesis that advanced-stage serous BOTs represent a distinct pathological entity compared with grade I serous epithelial ovarian carcinoma.

INTRODUCTION

In 1929, Taylor (1) first described a group of “semimalignant” ovarian tumors that had a more favorable prognosis than ovarian carcinoma. Characterized by complex branching papillae, epithelial stratification, nuclear atypia, mitotic activity, and absence of stromal invasion, these tumors are classified as low malignant potential or BOTs (2). The most common BOTs are serous, comprising ~55–60% of all BOTs; the others are mucinous, seromucinous, clear cell, mixed epithelial, or Brenner tumors (3). BOTs present at stages earlier than invasive ovarian epithelial cancer have a more indolent long-term course and excellent prognosis after surgical management alone (4–11). Sixty to 85% of patients present with stage I disease can be managed conservatively with unilateral salpingo-oophorectomy and complete staging. In some cases ovarian cystectomy may be performed, although these tumors are more likely to recur (5, 12). Posttreatment 5-, 10-, 15-, and 20-year survival rates are 99–100, 93–99, 90–95, and 88–95%, respectively, for stage I disease (7, 8, 10, 11). For the 15–30% of patients who present with stage II–III disease, TAHBSO and surgical staging are the treatments of choice. Approximately 10–30% of these patients will relapse, and 10% will die of the tumor. The roles of adjuvant chemotherapy and radiation are limited (6, 7, 9–11, 13, 14). An estimated 15% of patients will develop epithelial ovarian cancer (15, 16). Whether these invasive secondary tumors represent progression of borderline to invasive disease or new secondary tumors arising in an at-risk population is controversial (17). We hypothesized that the serous BOT is a precursor lesion, as a villous adenoma is a precursor to colon cancer. In the model of colon carcinogenesis, benign epithelium progressively accumulates mutations to become a carcinoma (18). A similar model has been proposed for epithelial ovarian cancer (19).

To better understand the potential relationship between these tumors, we compared K-ras and p53 mutations in tumors from eight patients who first presented with advanced-stage serous BOT and later developed grade I serous carcinomas. K-ras, a proto-oncogene located on chromosome 12, encodes a membrane-bound GTPase that stimulates signal transduction. K-ras mutations are a common feature of BOTs (3) and may play a role in tumor progression (20). p53 is a tumor suppressor gene encoding a 393-amino acid nuclear phosphoprotein thought to play a regulatory role in the cell cycle (21). Overexpression of p53 in serous BOTs is associated with an increased likelihood of progression or recurrence (22). Mutations of the p53 gene are infrequent in late stage and are extremely rare to absent in stage I BOTs, but are found with increasing frequency in serous carcinomas (23–25). The patients in this study underwent surgical resection of both the primary and secondary tumors. A limited number of specimens were available for molecular genetic analysis.

MATERIALS AND METHODS

Patients. The patients had a mean age of 45.7 years (age range, 35.3–59.5 years) at the time of initial diagnosis and surgery. Seven patients underwent TAHBSO and complete staging. One patient underwent unilateral salpingo-oophorectomy at the time of initial diagnosis. The interval between the diagnosis of a serous BOT and development of invasive serous epithelial ovarian carcinoma was 7–85 months (Table 1). Tissue from the primarily involved ovary removed at the first surgery was used for analysis. For the second surgery, tumor samples came from either small- or large-bowel resections in six cases (usually sigmoid or small bowel from the pelvis), the appendix in one case, and the spleen in one case.

Laser-Capture Microdissection. H&E-stained paraffin-embedded tissues were reviewed (by E. S. or M. D.) and selected for microdissection. A PixCell II Microscope was used to microdissect tumor epithelium from the surrounding stroma (Fig. 1; Ref. 26). The microscope was fitted with a laser beam focused on the slide through a thermoplastic polymer film bonded to a microcentrifuge tube cap. The laser was fired, annealing the cells to the film, which was digested along with cellular proteins to yield DNA for SSCP-PCR. Laser spot sizes were 7–10 mm with a pulse duration of 500–900 ms. Five thousand to 10,000 laser pulses obtained 10,000–50,000 epithelial cells. DNA was extracted with 50 µl of a digestion buffer containing 0.04% Proteinase K, 10 mM...
Tris-HCl (pH 8.0), 1 mM EDTA, and 1% Tween 20. Tubes were inverted to allow the digestion buffer to contact the cap. Samples were incubated overnight at 37°C and centrifuged for 5 min. After the cap and polymer were discarded, the tubes were heated to 95°C for 8 min to inactivate the protease K. Extracted DNA was quantified at 15–500 ng/μL by fluorometry (PICO Green, ds DNA Quantitation Kit; Molecular Probes, Inc., Eugene, OR) and used directly as a template for PCR.

SSCP-PCR Analysis and Sequencing. K-ras and p53 gene mutations were examined by SSCP-PCR analysis. K-ras codons 1–36 and 38–80 and p53 exons 5–11 were amplified from the tumor DNA by PCR with primers flanking the gene sequences. DNA was denatured at 94°C for 10 min. PCR amplification was carried out with 35 cycles of denaturation at 94°C for 1 min, annealing at 55–62°C (depending on the primer) for 1 min, and extension at 72°C for 2 min. The PCR products were then analyzed on a 6% polyacrylamide gel run at 30 W and 4°C for 2.5–4 h. DNA with altered mobility on SSCP analysis was eluted from gels and reamplified using the same primers and PCR conditions. The PCR product was then purified on a 1.2% agarose gel. DNA bands were cut from the gel, heated to 65°C for 30 min in 100 μL of distilled, purified water, and then purified using Sephaglas BandPrep DNA purification kit (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). Sequencing was performed using the Thermo Sequenase cycle sequencing kit (Amersham Life Science). Samples were preheated to 72°C for 2–10 min and run on a 6% polyacrylamide gel, containing 7 M urea, at 1800 V at room temperature.

The SSCP-PCR produced distinct DNA bands (Fig. 2). Contamination of the tissue by surrounding tissues such as stroma, blood vessels, or lymphatics was unlikely because laser-capture microdissection was used to obtain DNA for analysis. Tumor stroma was dissected and used as an internal control. The SSCP was repeated two to three times for each specimen, both to confirm the presence of mutations and to rule out the possibility of PCR-related artifacts. Sequencing was performed on the DNA eluted from the shifted SSCP bands.

RESULTS

Eight patients underwent primary surgical staging. Four underwent cytoreduction to zero visible disease. Three cases had <2 cm residual disease, and one case had >2 cm residual disease. For seven of eight (88%) patients, p53 mutations were completely different in the primary compared with the secondary tumor. The changes in nucleotide sequence and amino acid changes are summarized in Tables 2 and 3.

In three of these cases (SM, LW, and HL), p53 mutations in the primary were altogether absent from the secondary tumor. SM had a K-ras mutation in the primary that was absent from the secondary tumor. LW had different K-ras mutations in the primary and secondary tumors, and HL had no K-ras mutations in the primary but did have one in the secondary tumor. LW had a short time interval between the first and second tumors, which raised the question of possible suboptimal debulking. But the mutational analysis demonstrated that none of the p53 or K-ras mutations identified in the primary tumor were present in the secondary tumor, providing strong evidence against a clonal origin of the two tumors.

In two patients (CM and EV), p53 mutations were completely different in the primary versus secondary tumors. For EV, TCC→TCT was a silent mutation: Ser→Ser in exon 9. Although CM had identical K-ras mutations in the primary and secondary tumors, the p53 mutations for numerous exons were different, pointing to a different clonal origin for the two tumors. EV had no K-ras mutations in either tumor.

In two patients (JK and OG), p53 mutations were present in the
secondary tumor that had been absent in the primary tumors. The same was true for \(K-ras\) mutations for OG. JK had no \(K-ras\) mutations in either tumor. The difference in mutations between primary and secondary tumors in these two patients could reflect either acquisition of oncogene mutations during tumor progression or tumor heterogeneity. One case, UT, had no \(p53\) mutations in the exons examined.

**DISCUSSION**

In seven of eight cases (88%), mutational analysis of \(p53\) demonstrated completely different mutations in the primary BOTs compared with the subsequent serous carcinomas. For the patient who underwent fertility-sparing surgery, the primary and secondary occurrences were distinct tumors. The three (38%) cases who had \(p53\) mutations unique to the primary tumor provide the most compelling evidence for a nonclonal origin of the second tumor. The \(p53\) data, enhanced by the \(K-ras\) data, suggest a nonclonal origin for the serous BOT compared with the subsequent grade 1 invasive serous epithelial ovarian carcinoma.

The study had several limitations. One limitation is that although \(p53\) mutations are uncommon in BOTs (22–24), \(p53\) mutations occurred with some frequency in the BOTs in the present study, which may have been related to the advanced stages of the BOTs studied. Another limitation is that the number of patients studied was small, in part because of the logistics of long-term patient follow-up. A third limitation is that the multifocality of metastatic disease was not addressed. There is evidence pointing to unifocality of metastatic disease in ovarian carcinomas (27–29) and \(p53\) mutations that occur prior to metastatic spread and remain closely conserved (30). In the present study, evaluation of multiple tumor implants was limited by tissue availability and is the object of ongoing investigations. The fourth limitation is that the DNA for sequencing was obtained from the SSCP-PCR gel rather than by direct sequencing. The sensitivity of SSCP is 80–90%. A disadvantage of direct sequencing is that deletions or insertions shift the whole sequencing profile. In the presence of a normal allele, it may not have been possible to read the sequence. Because the SSCP was repeated several times, the presence of artifacts was less likely.

Our observations do not support the hypothesis that a serous BOT is a precursor lesion to invasive serous carcinoma. Molecular genetic analyses comparing the two tumors have attempted to identify common mutational or other events to establish a continuum from BOT to invasive carcinoma (4, 21, 24, 31, 32). Studies of \(K-ras\) in our laboratory demonstrated that although mucinous borderline tumors might be precursors, it was less clear for serous

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**Table 2: \(p53\) mutations in primary and secondary tumors**

Changes in nucleic acid bases reflect point mutations. \(K-ras\) codons 1–36 and 38–80 were examined.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Exon</th>
<th>Sequence change</th>
<th>Amino acid change</th>
<th>Exon</th>
<th>Sequence change</th>
<th>Amino acid change</th>
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<tr>
<td>SM</td>
<td>5</td>
<td>GGT→GTG</td>
<td>Val→Ile</td>
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<td></td>
<td>6</td>
<td>GAC→GTC</td>
<td>Asp→Val</td>
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<td></td>
<td>10</td>
<td>GCT→ACT</td>
<td>Ala→Thr</td>
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<td>Absent</td>
<td>Absent</td>
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<tr>
<td>HL</td>
<td>6</td>
<td>GG→GA</td>
<td>Gly→Arg</td>
<td>9</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>GG→GA</td>
<td>Intron</td>
<td>7</td>
<td>AAG→AAA</td>
<td>Asn→Lys</td>
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<tr>
<td></td>
<td>10</td>
<td>TTG→TTA</td>
<td>Leu→Leu</td>
<td>10</td>
<td>Absent</td>
<td>Absent</td>
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<tr>
<td>EV</td>
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<td>TCC→TCT</td>
<td>Ser→Ser</td>
<td>5</td>
<td>TAC→GAC</td>
<td>Tyr→Asp</td>
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<td></td>
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<td>Absent</td>
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<td>Arg→His</td>
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<tr>
<td>JK</td>
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<td>Absent</td>
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<td>GGG→GAG</td>
<td>Gly→Glu</td>
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<td>Glu→Glu</td>
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<tr>
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<td>TCT→TTT</td>
<td>Ser→Phe</td>
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<tr>
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<td>Absent</td>
<td>Absent</td>
<td>9</td>
<td>GGA→GAA</td>
<td>Gly→Arg</td>
</tr>
</tbody>
</table>

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**Table 3: \(K-ras\) mutations present in primary and secondary tumors**

Changes in nucleic acid bases reflect point mutations. \(K-ras\) codons 1–80 were examined.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Exon</th>
<th>Sequence change</th>
<th>Amino acid change</th>
<th>Exon</th>
<th>Sequence change</th>
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<td>LW</td>
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<td>Gly→Val</td>
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<td>Ala→Val</td>
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<tr>
<td>OG</td>
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<td>GGT→GTG</td>
<td>Gly→Val</td>
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<td>Gly→Val</td>
</tr>
<tr>
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<td>GGT→GTG</td>
<td>Gly→Val</td>
<td>3</td>
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<td>Gly→Val</td>
</tr>
<tr>
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<td>Gly→Val</td>
<td>3</td>
<td>GGT→GTG</td>
<td>Gly→Val</td>
</tr>
<tr>
<td>JK</td>
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<td>GGT→GTG</td>
<td>Gly→Val</td>
<td>3</td>
<td>GGT→GTG</td>
<td>Gly→Val</td>
</tr>
</tbody>
</table>

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**Fig. 2. SSCP-PCR.** SSCP-PCR gel from patient CM demonstrates \(p53\) mutations in exons 5 (a) and 7 (b). a, mutation in the epithelium of the primary tumor that was absent from the secondary tumor. b, mutation in the secondary tumor that was absent from the primary. Arrows indicate shifted bands. Stroma was used as an internal control: PE, primary tumor epithelium; PS, primary tumor stroma; SE, secondary tumor epithelium; SS, secondary tumor stroma.
K-ras AND p53 MUTATIONS IN SEROUS BOTs AND OvARian CANCERS

BOTs (4). Others have studied K-ras mutations in serous BOTs compared with grade 1 and 3 carcinomas. On the basis of the number of K-ras mutations in carcinoma versus BOTs, they concluded that BOTs were not precursor lesions (31, 32). These data support the conclusion that the serous BOTs and invasive serous carcinoma are unique entities.

Overexpression of p53 in serous BOTs has been associated with increased probability of progression or recurrence and decreased overall survival (21). Patterns of p53 immunoreactivity for BOTs have been compared with malignant epithelial ovarian tumors, and some have concluded that the tumors were distinct biological entities (26). p53 mutations are rare in BOTs, and mutational analysis has yielded conflicting results (23, 24).

Studies of LOH have revealed similar patterns of LOH in early- and late-stage malignant tumors, suggesting that the evolution of BOTs and carcinomas follows similar patterns of genetic alteration (33, 34). However, common chromosomal inactivation patterns have not been reported universally. Some authors have reported that LOH on chromosome 17 may not be as important in BOT pathogenesis as in true carcinomas (35). Using comparative genomic hybridization, Wolf et al. (36) agreed that different mechanisms may lead to BOT formation. Although K-ras mutational patterns may be similar for the tumors, Haas et al. (34) noted that microsatellite instability data suggest that serous carcinoma and BOTs are in fact distinct tumors.

These data point to a growing consensus that the serous BOTs and carcinoma do not represent a continuum of tumor progression. It may be that the conflicting data reflect subtypes of serous BOTs: those that progress, and those that do not. A "micropapillary" subtype of serous BOT that is more likely to be associated with advanced stage, recurrence, and a poorer prognosis has been described (37). The tumors in this study were analyzed based on the traditional classification of serous BOTs and were not subclassified retroactively.

This study is the first, to our knowledge, in which within-subject comparative mutational analysis was performed. The data suggest that the semimalignant tumors first described by Taylor (1) and later classified as part of the spectrum of ovarian neoplasia are unlikely to be precursors of invasive epithelial ovarian cancers. Further study of micropapillary BOTs may elucidate the relationship, if any, between the serous BOT and serous epithelial ovarian cancer.

REFERENCES

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