Detection of Residual Subclinical Ovarian Carcinoma after Completion of Adjuvant Chemotherapy

Mory Nouriani,1 Afshin Bahador,1 Jonathan S. Berek,4 Joan P. Cheng,5 Dennis S. Chi,5 William A. Ciliby,7 Giuseppe Del Priore,8 Mark K. Dodson,9 Bridgette D. Duggan,10 David M. Gershenson,11 Scott E. Lentz,1 Richard T. Penson,12 William R. Robinson,5 Mildred Rodriguez,1 Lynda D. Roman,1 Mimi C. Yu,1 Karen Zempolich,9 and Louis Dubeau2

1Division of Gynecologic Oncology and Departments of 2Pathology and 3Preventative Medicine, University of Southern California Keck School of Medicine, Los Angeles, California; 4Division of Gynecologic Oncology, University of California, Los Angeles School of Medicine, Los Angeles, California; 5Division of Gynecologic Oncology, Tulane Medical School, New Orleans, Louisiana; 6Department of Surgery, Memorial Sloan Kettering Cancer Center, New York, New York; 7Section of Gynecologic Surgery, Mayo Clinic, Rochester, Minnesota; 8New York University School of Medicine, New York, New York; 9Department of Obstetrics and Gynecology, University of Utah School of Medicine, Salt Lake City, Utah; 10Gynecologic Oncology Associates, La Jolla, California; 11Department of Gynecologic Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas; and 12Divisions of Hematology-Oncology and Gynecologic Oncology, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts

ABSTRACT

Purpose: We sought to test the hypothesis that the presence of telomerase activity in peritoneal washings of patients treated for ovarian carcinoma is a sensitive and specific indicator of the presence of residual disease. We hypothesized that this test, if added to second-look procedure protocols, could help determine whether residual disease is present or not in patients who have completed their adjuvant chemotherapy for ovarian carcinoma.

Experimental Design: Peritoneal washings were obtained from 100 consecutive patients undergoing a second-look procedure after treatment for ovarian carcinoma (cases) and from 100 patients undergoing surgery for benign gynecological conditions (controls). The washings were assayed for telomerase activity using the telomerase repeat amplification protocol. The results were compared to the histological and cytological findings.

Results: Among our 100 cases, 82 (82%) had either positive second-look procedures or expressed telomerase in their peritoneal washings. Fifty-three (53%) had positive second-look procedures, whereas 66 (66%) tested positive for telomerase. Twenty-nine of the 47 patients (62%) with negative second-look procedures tested positive for telomerase. Of the 53 patients with positive second-look procedures, 37 (70%) tested positive for telomerase. None of the 100 controls (0%) expressed telomerase in their peritoneal washings.

Conclusions: Telomerase activity in peritoneal washings of patients treated for ovarian carcinoma and undergoing a second-look procedure may provide a means of increasing the sensitivity of such procedures for the detection of residual disease while maintaining a high level of specificity.

INTRODUCTION

No reliable method exists for accurately documenting absence of residual disease in patients treated for epithelial ovarian carcinoma. Posttherapy surveillance using computed tomography scanning or ultrasound imaging has yielded high false-positive and false-negative rates (1–3). Although an elevated CA125 is highly indicative of the presence of residual disease in this patient population (4–6), serial measurements of this circulating antigen have a sensitivity of only 40% in detecting residual ovarian carcinoma (5, 7, 8).

Of all current surveillance modalities, second-look procedures provide the most accurate assessment of response to chemotherapy in patients with advanced epithelial ovarian cancer (9–11). These procedures refer to laparotomies or laparoscopies, usually performed 6 weeks after completion of chemotherapy, in patients who display no clinical evidence of residual disease. After obtaining peritoneal washings, the abdomen and pelvis are methodically explored. The peritoneum overlying the cul de sac and para-colic gutters, as well as all adhesions to the bowel, are biopsied. Residual omentum and ovarian pedicles are removed. If applicable, an appendectomy is performed, and residual tumor nodules are resected. In the past, second-look procedures were widely used to aid physicians in deciding whether to stop, change, or continue chemotherapy in patients undergoing treatment. However, the fact that up to 50% of patients in whom no residual carcinoma was detected during such procedures subsequently developed disease recurrence (9, 12, 13) has prompted most centers to abandon these procedures except when mandated by research protocols (9, 14–17).
Recent advances in our understanding of the molecular biology of cancer have raised the prospects of using diseasespecific molecular markers as tools for cancer detection. Telomerase, an enzyme responsible for maintaining the length of telomeres (18), may be particularly attractive in that regard. Telomeres are tandem repeats of a short DNA sequence at the end of each chromosome. The length of telomeres decreases in somatic cells each time they divide. Most mammalian cells are therefore limited as to the number of times they can undergo cell division because chromosomes cannot replicate unless their telomeres are longer than a minimal critical length (19–21). This mitotic clock does not operate in cancer cells because almost all cancers express telomerase, ensuring maintenance of the telomeres above the critical length necessary to support cell division (22, 23).

We reported previously that the presence of telomerase activity in peritoneal washings is an accurate indicator of the presence of disease in women undergoing primary surgery for ovarian carcinoma (24). When we compared the sensitivity of this approach with that of cytological examination of the same washings, the telomerase assay was found to be more sensitive than the cytology gold standard (87% sensitivity versus 64% sensitivity for cytology; Ref. 24). These findings prompted us to initiate a multi-institutional prospective study to evaluate the potential utility of assaying for telomerase as a tool for detecting the presence of residual disease in patients undergoing secondlook procedures after treatment for ovarian cancer. This paper compares the outcome of a telomerase assay performed on peritoneal washings from the first 100 patients enrolled in this prospective study with the surgical and pathological findings obtained during their second-look procedures.

MATERIALS AND METHODS

Study Patients. Subjects were enrolled from a larger, ongoing study involving nine participating medical centers. The first 100 consecutive cases and controls meeting eligibility criteria were included in this study. All cases underwent secondlook laparotomy or laparoscopy for serous or endometrioid ovarian carcinoma. None had clinical evidence of disease before the second look procedure based on physical examination, circulating CA125 levels, and radiological findings. Controls were from 100 subjects undergoing laparotomy or laparoscopy for noncancerous gynecological conditions at the Los Angeles County-University of Southern California Women’s and Children’s Hospital. The women in the control arm had no prior history of malignancy. All protocols were approved by institutional review boards at all collaborating institutions.

Collection of Specimens. All fluids obtained intraoperatively were collected immediately after the initial surgical incision and before exploration of the peritoneal cavity was initiated. Washings were obtained from the pelvis, right and left para-colic gutters, and diaphragm in all cases. Samples collected at institutions outside Los Angeles were either shipped overnight to the University of Southern California or centrifuged at 4000 × g for 20 min and later shipped to Los Angeles as frozen pellets. Samples from 20 different patients were processed using both of these methods for comparison of the two protocols.

**MATERIALS AND METHODS**

Study Patients. Subjects were enrolled from a larger, ongoing study involving nine participating medical centers. The first 100 consecutive cases and controls meeting eligibility criteria were included in this study. All cases underwent second-look laparotomy or laparoscopy for serous or endometrioid ovarian carcinoma. None had clinical evidence of disease before the second look procedure based on physical examination, circulating CA125 levels, and radiological findings. Controls were from 100 subjects undergoing laparotomy or laparoscopy for noncancerous gynecological conditions at the Los Angeles County-University of Southern California Women’s and Children’s Hospital. The women in the control arm had no prior history of malignancy. All protocols were approved by institutional review boards at all collaborating institutions.

Collection of Specimens. All fluids obtained intraoperatively were collected immediately after the initial surgical incision and before exploration of the peritoneal cavity was initiated. Washings were obtained from the pelvis, right and left para-colic gutters, and diaphragm in all cases. Samples collected at institutions outside Los Angeles were either shipped overnight to the University of Southern California or centrifuged at 4000 × g for 20 min and later shipped to Los Angeles as frozen pellets. Samples from 20 different patients were processed using both of these methods for comparison of the two protocols.

**Materials and Methods**

Study Patients. Subjects were enrolled from a larger, ongoing study involving nine participating medical centers. The first 100 consecutive cases and controls meeting eligibility criteria were included in this study. All cases underwent second-look laparotomy or laparoscopy for serous or endometrioid ovarian carcinoma. None had clinical evidence of disease before the second look procedure based on physical examination, circulating CA125 levels, and radiological findings. Controls were from 100 subjects undergoing laparotomy or laparoscopy for noncancerous gynecological conditions at the Los Angeles County-University of Southern California Women’s and Children’s Hospital. The women in the control arm had no prior history of malignancy. All protocols were approved by institutional review boards at all collaborating institutions.

Collection of Specimens. All fluids obtained intraoperatively were collected immediately after the initial surgical incision and before exploration of the peritoneal cavity was initiated. Washings were obtained from the pelvis, right and left para-colic gutters, and diaphragm in all cases. Samples collected at institutions outside Los Angeles were either shipped overnight to the University of Southern California or centrifuged at 4000 × g for 20 min and later shipped to Los Angeles as frozen pellets. Samples from 20 different patients were processed using both of these methods for comparison of the two protocols.

**Fig 1** Example of a positive telomerase repeat amplification protocol assay in peritoneal washings from a patient undergoing second-look laparotomy. Peritoneal washings (total volume, 50 ml) were centrifuged, and a protein extract was obtained from the resulting pellet. Telomerase activity was assayed using the telomerase repeat amplification protocol method (20). The radiolabeled reaction products were electrophoresed on 8% polyacrylamide gels and visualized by autoradiography. The presence of a characteristic ladder in Lanes A and C indicates the presence of telomerase activity. Lane A, test sample; Lane B, test sample preincubated with RNase A to inactivate telomerase, a ribonucleoprotein; Lane C, test sample supplemented with small amounts of a telomerase-positive cell extract.

**Assay for Telomerase Activity.** Cell pellets from peritoneal washings were lysed and tested for the presence or absence of telomerase activity using the telomerase repeat amplification protocol (TRAP; Ref. 23) assay as described previously (25). The reaction products were electrophoresed on 8% polyacrylamide and visualized by autoradiography. Telomerase products, when present, appeared as a characteristic ladder on the autoradiograph (Fig. 1), corresponding to DNA fragments differing in sizes by 6 bp, the length of the basic telomeric sequence. At least three consecutive bands needed to be present for a test to be called positive (Fig. 1). Each assay was performed in parallel with two control reactions, one in which the assay was preceded by digestion of the sample with RNase A (catalog number R5503; Sigma Chemical Co., St. Louis, MO) at a final concentration of 0.05 mg/ml, and another in which 1 μl of a cell lysate previously shown to contain high levels of telomerase activity was added to the test sample. A sample was not scored as positive unless the RNase A-treated control showed complete absence of telomerase products because telomerase is a riboprotein that is inactivated by RNase A. Conversely, a sample was not scored as negative unless products were detected after addition of a telomerase-positive extract to that sample (25).

**Statistical Analyses.** We used the two-sample t test to compare the mean ages between cases and controls. We used the nonparametric Wilcoxon rank-sum test to compare cases and controls in terms of gravidity and parity. Fisher’s exact test was used to compare the prevalence of positive second-look procedures and positive TRAP assays between cases and controls. We used the one-sample binomial test (null hypothesis, P = 0.5) to examine the concordance in results of the TRAP assay between two different methods of processing peritoneal washings before analysis (26). All Ps quoted are two-sided. Ps < 0.05 are considered statistically significant.
RESULTS

Study Participants. A total of 113 consecutive subjects with ovarian carcinoma were initially considered in this study, 13 of whom were excluded because of delays of >24 h from the time the sample was collected to the time of receipt of the unrefrigerated specimen in Los Angeles. One hundred and three control subjects also were considered. Three were excluded because of incidental findings of ovarian tumors of low malignant potential on subsequent examination of their pathological specimens.

Characteristics of the 100 subjects and 100 controls used in the study are shown in Table 1. There were no significant differences between these two groups except for the lower average age of the control group (43.9 years compared with 53.4 years for the cases). These age differences were expected, given our selection criteria for the two groups of patients. All together, 66 of 100 cases (66%) expressed telomerase activity in peritoneal washings, whereas none of the 100 control subjects (0%) expressed such activity based on results of the TRAP assay, attesting to the high specificity of this enzyme activity for the cancer phenotype in this experimental setting (Table 1).

Stability of Telomerase in Unrefrigerated Samples Shipped Overnight from Participating Institutions. Several washing specimens collected at non-University of Southern California-affiliated institutions were shipped via an overnight mail carrier in the absence of refrigeration. We sought to verify the merit of this approach. Although we had reported previously that telomerase activity remained stable for up to 5 days in specimens left at room temperature in the laboratory (24), we needed to verify whether this would also be true for specimens subjected to the extremes in temperature fluctuation that invariably occur during routine overnight air shipments. Fig. 2 shows the results of a TRAP assay performed on washing specimens from pelvis, right and left para-colic gutters, and diaphragm of a patient enrolled at Tulane University. All four washings were shipped to the University of Southern California via overnight mail without refrigeration. The presence of the characteristic ladder in all lanes after autoradiography of the products of the TRAP assay (Fig. 2) indicates that telomerase activity had remained stable in these samples despite the wide temperature fluctuations that inevitably took place during the shipping process.

We compared our two methods of processing peritoneal washings (overnight shipment without refrigeration versus shipment as frozen cell pellets) in 20 patients from whom both types of specimens were collected. There was 100% concordance between the results of the two sets of TRAP assays ($P < 0.0001$; binomial test, null hypothesis, $P = 0.5$). We conclude that overnight shipment of unrefrigerated samples did not result in significant loss of telomerase activity in peritoneal washings and constitutes a reliable method of sample collection.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Clinicopathological characteristics of our study population</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cases ($n = 100$)</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>$53.4 \pm 10.1^a$</td>
</tr>
<tr>
<td>Gravidy</td>
<td>$2.4 \pm 1.9$</td>
</tr>
<tr>
<td>Parity</td>
<td>$2.0 \pm 1.6$</td>
</tr>
<tr>
<td>No. of laparoscopy (%)</td>
<td>$13 (13)$</td>
</tr>
<tr>
<td>No. of positive TRAP$^b$ results (%)</td>
<td>$66 (66)$</td>
</tr>
<tr>
<td>Diagnosis</td>
<td></td>
</tr>
<tr>
<td>Serous carcinoma</td>
<td>89</td>
</tr>
<tr>
<td>Endometroid carcinoma</td>
<td>11</td>
</tr>
<tr>
<td>Leiomyoma</td>
<td>61</td>
</tr>
<tr>
<td>Endometriosis</td>
<td>2</td>
</tr>
<tr>
<td>Adenomyosis</td>
<td>9</td>
</tr>
<tr>
<td>Dermoid tumor</td>
<td>13</td>
</tr>
<tr>
<td>Paratubal cyst</td>
<td>5</td>
</tr>
<tr>
<td>TOA/hydropsaplinx</td>
<td>5</td>
</tr>
<tr>
<td>Tubal ligation</td>
<td>4</td>
</tr>
<tr>
<td>Serous cystadenoma</td>
<td>7</td>
</tr>
<tr>
<td>Mucinous cystadenoma</td>
<td>4</td>
</tr>
</tbody>
</table>

$^a$ Data presented as mean ± SD.

$^b$ TRAP, telomerase repeat amplification protocol; NA, not applicable; TOA, tubo-ovarian abscess.
Telomerase Assay for Residual Ovarian Cancer Detection

Our results clearly show that a substantial number of patients with no other evidence of residual disease at the time of their second-look procedures showed telomerase activity in peritoneal washings. The fact that none of our control patients showed such enzyme activity in similar washings supports our hypothesis that this test might increase our ability to identify residual disease in patients treated for ovarian carcinoma, although final proof of this idea awaits our ability to identify residual disease in patients treated for ovarian carcinoma.

DISCUSSION

Our results clearly show that a substantial number of patients with no other evidence of residual disease at the time of their second-look procedures showed telomerase activity in peritoneal washings. The fact that none of our control patients showed such enzyme activity in similar washings supports our hypothesis that this test might increase our ability to identify residual disease in patients treated for ovarian carcinoma, although final proof of this idea awaits our ability to identify residual disease in patients treated for ovarian carcinoma.

Fig. 3 Comparison between the outcome of the telomerase repeat amplification protocol assay and that of second-look procedures in 100 consecutive patients with ovarian carcinoma. *, a positive second-look procedure (SLL) was defined as a procedure with either histological or cytological evidence of persistent disease.

Discordances between the Results of the TRAP Assay and Pathological Findings. Forty-seven of our 100 subjects showed no evidence of residual disease at the time of their second-look procedures based on histological and cytological findings. As shown in Fig. 3, 29 of these 47 patients (61.7%) expressed telomerase activity in peritoneal washings obtained during this procedure. Conversely, histological or cytological evidence of residual disease was found in 53 of our 100 subjects. Of these 53 patients, 37 (69.8%) also tested positive for telomerase. The remaining 16 patients (30.2%) showed no detectable telomerase activity in their peritoneal washings. In 10 such cases, the disease was microscopic. In the most extreme case, the residual disease was confined to a few microscopic tumor cells of unclear viability in the center of a 6-cm necrotic mass. In three of the remaining six patients, the tumor was outside the peritoneal cavity, either in the retroperitoneum or above the fascia in the s.c. tissue. Of the remaining three patients, one had 1-cm plaques on the bowel surface, and two had military stud-ding, which was confined to the bowel mesentery in one case and present throughout the abdomen in the other case.

The laparotomy/laparoscopy findings in our patient population are consistent with those of previously published studies. Forty-seven percent of patients undergoing a second-look procedure in our study had no evidence of disease, consistent with the 35–50% negative second-look rate quoted by other investigators (27–29). Although follow-up information is not yet available for our study population, it is interesting that the proportion of patients with negative second-look procedures who demonstrated telomerase activity in their peritoneal fluid, which is 62%, is close to the 50% rate of eventual recurrence reported in patients who demonstrate no evidence of disease at second-look laparotomy (9, 12, 13).

Nearly one-third of patients with pathological evidence of disease at the time of their second-look procedure tested negative for telomerase. The majority of these cases showed microscopic tumor cells, which in some cases were found outside the peritoneal cavity. Nevertheless, cytology was positive in 8 of these 16 patients. These results suggest that the telomerase assay should be most powerful when used in conjunction with a second-look procedure. Eighty-two of our 100 patients were positive for either the second-look procedure or the telomerase assay. These numbers are consistent with previous reports that up to 75% of patients with advanced epithelial ovarian carcinoma develop recurrence, even after optimal treatment (30, 31).

Potential reasons for the apparent failure of the telomerase assay to detect residual cancer cells in some patients shown to harbor i.p. disease based on surgical findings, such as our three telomerase-negative patients with macroscopic tumor, include the presence of reaction inhibitors or inactivation of telomerase due to mishandling of the washing samples. The observation that some cancers, particularly those with complete inactivation of the p53 pathway (32), occasionally use means other than telomerase expression to maintain the length of their telomeres raises the possibility that some of our telomerase-negative, second-look-positive subjects had tumors that did not express possibility that the peritoneal irritation that resulted from these interventions accounted for the observed increase in telomerase expression in our cases cannot be entirely ruled out, the presence of conditions associated with peritoneal irritation and repair in some of our controls, such as, for example, endometriosis, argues against this possibility. In addition, the fact that a positive correlation between TRAP positivity and stage or extra-ovarian tumor volume was observed in a previous study from our laboratory (24), in which 6 of 14 (42%) of patients with no or small extraovarian disease tested positive for this assay compared with 21 of 28 (75%) of those with extraovarian disease larger than 2 cm, further alleviates this concern.

This approach is attractive for use in clinical protocols for several reasons. First, because most ovarian cancers express telomerase, it is applicable to the great majority of patients affected by this disease (25). Second, the telomerase enzyme is stable in unprocessed and unrefrigerated specimens, greatly facilitating the use of this test in clinical settings. Finally, this approach is based on the presence of an enzyme activity, implying that a positive test is likely to indicate the presence of viable cancer cells as opposed to cancer cell debris. This last point is important because such debris are undoubtedly abundant in the peritoneal cavity of patients treated for ovarian carcinoma.

The laparotomy/laparoscopy findings in our patient population are consistent with those of previously published studies. Forty-seven percent of patients undergoing a second-look procedure in our study had no evidence of disease, consistent with the 35–50% negative second-look rate quoted by other investigators (27–29). Although follow-up information is not yet available for our study population, it is interesting that the proportion of patients with negative second-look procedures who demonstrated telomerase activity in their peritoneal fluid, which is 62%, is close to the 50% rate of eventual recurrence reported in patients who demonstrate no evidence of disease at second-look laparotomy (9, 12, 13).

Nearly one-third of patients with pathological evidence of disease at the time of their second-look procedure tested negative for telomerase. The majority of these cases showed microscopic tumor cells, which in some cases were found outside the peritoneal cavity. Nevertheless, cytology was positive in 8 of these 16 patients. These results suggest that the telomerase assay should be most powerful when used in conjunction with a second-look procedure. Eighty-two of our 100 patients were positive for either the second-look procedure or the telomerase assay. These numbers are consistent with previous reports that up to 75% of patients with advanced epithelial ovarian carcinoma develop recurrence, even after optimal treatment (30, 31).

Potential reasons for the apparent failure of the telomerase assay to detect residual cancer cells in some patients shown to harbor i.p. disease based on surgical findings, such as our three telomerase-negative patients with macroscopic tumor, include the presence of reaction inhibitors or inactivation of telomerase due to mishandling of the washing samples. The observation that some cancers, particularly those with complete inactivation of the p53 pathway (32), occasionally use means other than telomerase expression to maintain the length of their telomeres raises the possibility that some of our telomerase-negative, second-look-positive subjects had tumors that did not express
telomerase. We are unable to test this possibility because fresh tumor tissues were not available from our study subjects. Whether or not lack of telomerase expression in the presence of histologically proven disease has any prognostic significance is presently unclear.

There are a number of ways in which the outcome of the telomerase assay could potentially influence the clinical management of patients with otherwise negative second-look procedures. Such women are typically evaluated by quarterly physical examinations during the first 2 years. During each visit, tumor markers such as CA125 are obtained. Patients who are telomerase positive may benefit from being followed at closer intervals. In addition, telomerase positivity may identify a subset of patients who are suitable candidates for salvage chemotherapy. The presence of gross disease during second-look procedures is a poor prognostic indicator (33–35). No salvage chemotherapy regimen has been shown to be effective in improving survival in this group of patients. However, several studies have shown that salvage or consolidation chemotherapy can confer a definite benefit in patients with microscopic disease detected during second-look procedures, with 5-year survival rates as high as 70% (35, 36). Given that patients with false-negative second-look procedures are likely to harbor microscopic disease, the prospects of identifying such patients, followed by administration of additional chemotherapy, may confer a better survival.

In summary, our results suggest that performance of the TRAP assay on peritoneal washings of patients treated for ovarian carcinoma, when used as an adjunct to the second-look procedure, could substantially improve the sensitivity of this procedure for the detection of residual cancer cells while maintaining a high degree of specificity. The question of whether or not patients with negative second-look procedures who test positive for telomerase are more likely to undergo clinical recurrence awaits the availability of follow-up data from our study population.

ACKNOWLEDGMENTS

We thank Dr. Arlan F. Fuller Jr. (Massachusetts General Hospital, Harvard Medical School) for critical review of the manuscript.

REFERENCES

Detection of Residual Subclinical Ovarian Carcinoma after Completion of Adjuvant Chemotherapy
