Detection of Ovarian Cancer Cells: Comparison of a Telomerase Assay and Cytologic Examination

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Background: Telomerase is an enzyme essential for the normal replication of chromosomes. Telomerase activity is absent in most somatic cells in adults, but it is usually expressed in cancer cells, including ovarian carcinoma cells. Our principal goal was to compare the sensitivity of a telomerase assay, i.e., the telomeric repeat amplification protocol (TRAP) assay, with that of cytologic examination in detecting cancer cells in the peritoneal cavity of patients with ovarian carcinoma. Methods: TRAP assays and cytologic examinations were performed on peritoneal washings and ascitic fluids from 42 patients with active ovarian carcinoma. Control specimens included washings from 29 patients with benign ovarian diseases and ascitic fluids from 14 patients with liver failure. We also evaluated the stability of telomerase in ascitic fluids left unprocessed at room temperature as well as the ability of the TRAP assay to detect cancer cells in mixtures containing large numbers of normal cells. Results: Specimens from 37 (88%) of the 42 patients with ovarian carcinoma tested positive for telomerase. Cytologic examination detected cancer cells in only 27 of the telomerase-positive specimens (i.e., in specimens from 64% of the 42 patients). This difference of 24% (95% confidence interval = 17%–30%) in sensitivity between the two tests was statistically significant (two-sided P = .002). Specimens from five of the patients with ovarian carcinoma were cytologically negative and telomerase negative. All 43 control specimens were cytologically negative, but the TRAP assay detected telomerase in two of them. Telomerase activity was detected in unprocessed samples left at room temperature for 5 days and in mixtures containing a small number of cancer cells and a 2000- to 10 000-fold excess of normal cells. Conclusions: Assaying for telomerase is more sensitive than cytologic examination in detecting cancer cells in the peritoneal cavity of patients with ovarian carcinoma. [J Natl Cancer Inst 1998;90:238–42]

Advanced ovarian carcinomas usually remain confined to the peritoneal cavity. Thus, the initial treatment, which consists of tumor reductive surgery and subsequent chemotherapy, was, until recently, often followed by a comprehensive surgical re-exploration of the pelvis and the abdomen to evaluate treatment response and to rule out the presence of residual disease. Patients with no macroscopic disease at the time of this second operation had biopsy specimens taken from multiple sites, following rigorous protocols, to rule out the presence of microscopic disease. Washings from various sites were also obtained and examined by a cytopathologist. Those patients in whom either gross or microscopic tumors were found were given additional chemotherapy. The remaining patients, who accounted for approximately 35% of those with ovarian cancer (1), received no further treatment. These surgical re-explorations, called “second-look laparotomies,” are still performed in some centers and are often part of experimental protocols. However, they are no longer part of routine management protocols in most institutions because of their low sensitivity. Indeed, although such procedures are clearly the most sensitive means of detecting residual disease after the completion of adjuvant chemotherapy for ovarian carcinoma, 24%–54% of patients in whom no residual tumor is found at the time of their second-look laparotomy experience later recurrences in spite of such negative findings (1). There is, therefore, a need for novel diagnostic techniques to improve our ability to detect residual disease in the peritoneal cavity for this group of patients.

Studies (2,3) demonstrating telomerase expression in most ovarian carcinomas encouraged us to evaluate the potential usefulness of assaying for this enzyme in washings or ascitic fluids from the peritoneal cavity as a means of detecting residual or recurrent cancer cells in patients treated for advanced ovarian carcinoma. Telomerase is a ribonucleoprotein enzyme responsible for maintaining telomere length in dividing cells (4–6). It is expressed in some normal cells, such as germ cells and stem cells, but it is not expressed in most somatic cells in the adult (7). Telomerase is thought to be important for the continuous growth of cancer cells because telomeres, which are essential for normal chromosome replication, undergo progressive shortening during each cell division. Indeed, telomerase is expressed in a large number of cancer cell types (7). We sought to compare the sensitivity of a telomerase assay, i.e., the telomeric repeat amplification protocol (TRAP) assay, with that of cytologic examination in the detection of cancer cells in the peritoneal cavity of patients with ovarian carcinoma. We also sought to evaluate the stability of telomerase and the sensitivity of this assay to assess further the potential clinical utility of this test.

Materials and Methods

Source of Pelvic–Peritoneal Fluid Samples

Ascitic fluids or peritoneal washings were collected from 42 patients undergoing exploratory laparotomy for primary or recurrent ovarian carcinoma and from 29 patients undergoing exploratory laparotomy for benign ovarian masses. Both groups of patients were treated at various institutions affiliated with the University of Southern California Medical School, Los Angeles. In addition, ascitic fluids were obtained from percutaneous paracenteses in 14 patients who had noncancerous liver disease. All pro-

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cedures were approved by an institutional review board and were performed after appropriate consents were obtained.

Of our 42 patients with ovarian carcinoma, 22 had papillary serous ovarian carcinomas, seven had endometrioid carcinomas, five had mucinous carcinomas, four had clear cell carcinomas, three had undifferentiated carcinomas, and one had a carcinosarcoma. Six of these tumors were assigned a histologic grade of 1, seven were assigned a grade of 2, and 29 were assigned a grade of 3. Tumor grading was done according to conventional criteria [see (8)]. Among the 29 patients with benign ovarian diseases, nine had serous cystadenomas, two had mucinous cystadenomas, one had an ovarian fibroid, one had teratomas (dermoid tumors), and six had endometriosis.

Diagnostic verification and tumor subtyping or grading were performed by one investigator (L. Dubeau). Cytologic pathologic evaluation of each fluid sample was reviewed independently, also by one investigator (S. E. Martin). These diagnostic evaluations were initially done in a blinded manner, i.e., without knowledge of the telomerase results. However, each cytologically negative case that showed measurable telomerase activity was later reviewed by the same cytopathologist (S. E. Martin) to increase the stringency of the cytologic evaluations. Surgical staging was done according to the current recommendations of the International Federation of Gynecology and Obstetrics [see (8)].

Source of Cell Lines and Strains

SKOV-3 and OVCAR-3 ovarian carcinoma cells (catalog numbers HTB77 and HTB161, respectively) were obtained from the American Type Culture Collection, Rockville, MD. HEY and HOC-7 ovarian carcinoma cells were obtained from Dr. Ronald Buick, University of Toronto (9). Normal human foreskin fibroblasts were isolated by use of published protocols (10).

Sample Processing and Telomerase Assay

All fluids obtained intraoperatively were collected immediately after the initial surgical incision and before exploration of the peritoneal cavity was initiated. Samples (50–500 mL) were centrifuged at 4000 g for 20 minutes at room temperature. The resulting cell pellets were rinsed with ice-cold washing buffer (10 mM HEPES–KOH [pH 7.5], 1.5 mM MgCl₂, 10 mM KCl, and 1 mM dithiothreitol), and protein extracts were prepared and assayed for telomerase by use of the TRAP assay as previously described (3). In each case, a control reaction in which the protein extract was treated with ribonuclease A at a final concentration of 0.05 mg/mL for 20 minutes at 37 °C before the TRAP assay (to inactivate telomerase) was run in parallel (3). For those extracts showing no telomerase activity, aliquots were mixed with extracts known to have telomerase activity (used at 1:100 dilutions) and re-assayed to rule out false-negative results caused by TRAP reaction inhibitors (3).

Statistical Analyses

The one-sample binomial test (null hypothesis: P = .5) conducted on discordant pairs was used to evaluate the possible difference in sensitivity or specificity between the TRAP assay and cytologic examination (11). Fisher’s exact test (11) was used to assess the difference in the rate of false-negativity in the telomerase assay between mucinous tumors and nonmucinous tumors. All reported P values are two-sided.

Results

Measurable Telomerase Activity in Peritoneal Washings and Ascitic Fluids From Patients With Ovarian Carcinomas

Initially, we determined the feasibility of measuring telomerase activity in either ascitic fluids or peritoneal washings from patients with ovarian carcinoma by use of the sensitive approach, i.e., the TRAP assay, which was developed by Kim et al. (7). We were concerned that potential enzyme inhibitors or, perhaps, degradative enzymes in these enzyme-rich fluids would interfere with this assay. However, 95% of our telomerase-negative samples showed activity when they were supplemented with small amounts of protein extracts known to contain active telomerase.

Although the exact nature of the suspected enzyme inhibitor(s) in the remaining 5% of our samples is not known, it does not appear to be a blood product, such as hemoglobin, because telomerase activity was readily detected in several of our blood-soiled samples.

Sensitivity of the TRAP Assay in Comparison With That of Cytologic Examination

We next tested the hypothesis that the TRAP assay is a more sensitive test than cytologic examination for the detection of cancer cells in peritoneal washings or ascitic fluids from patients with ovarian carcinoma. Therefore, we obtained such specimens from 42 patients undergoing surgical exploration for ovarian carcinoma and in whom the presence of either gross or microscopic tumor was confirmed histologically. All samples were examined by a cytopathologist (S. E. Martin) and were tested with the use of the TRAP assay in a double-blinded fashion. Following this initial examination, all cases that tested positive for telomerase but were interpreted as negative by cytologic examination were brought to the attention of the same cytopathologist and re-evaluated to increase the stringency of the cytologic data.

The cytologic findings and the results of the telomerase assay for all of the case patients are shown in Table 1. The results of the two tests were concordant in 32 cases, 27 of which were positive and five of which were negative. All 10 discordant cases were scored positive by the telomerase assay but negative by cytologic examination.

Additional clinico-pathologic parameters for the 42 patients are also listed in Table 1. The telomerase assay showed positive results for all three patients who had no histologic evidence of tumor spread outside their ovaries, i.e., individuals with stage IC disease (cytologically positive washings or ascites, tumor on the ovarian surface, or tumor confined to the ovaries that was accidentally ruptured or spilled at the time of surgery). One of these patients had ascites and was positive by cytologic examination. The other two patients were cytologically negative. Nonetheless, peritoneal washings from these latter two patients would be expected to contain dislodged ovarian carcinoma cells. Thus, the sensitivity of the TRAP assay was 88% compared with 64% for cytologic examination. This difference of 24% (95% confidence interval [CI] = 17%–30%) in sensitivity between the two tests was statistically significant (P = .002).

The seven telomerase-positive patients among 10 patients with recurrent tumors included three patients in whom the tumor was identified by microscopic examination only, i.e., no gross evidence of disease at the time of the surgery) and one patient in whom the tumor was less than 1 cm in diameter. These results further emphasize the sensitivity of the telomerase assay as well as its potential usefulness in documenting recurrences of small tumors.

Table 1 suggests a larger degree of discordance between the telomerase assay and cytologic examination in specimens obtained from washings than in specimens obtained from ascitic fluids. A greater degree of discordance was also seen when findings from patients with either small or histologically undetectable extra-ovarian disease were compared with findings from patients with large (≥2 cm) extra-ovarian tumors (five of 14 versus five of 28), underscoring the potential usefulness of the telomerase assay in patients with minimal disease that is difficult to detect by conventional methods.
Finally, the specimens were subdivided into those obtained from patients with normal versus elevated serum levels of CA125, since this antigen is frequently expressed by ovarian cancer cells and is often used clinically to monitor disease recurrence following treatment (12). Although most of the patients examined showed elevated levels of CA125 (>35 U/mL), two of the four patients with normal levels (<35 U/mL) tested positive for telomerase (Table 1), suggesting that the telomerase assay could complement measurement of CA125 for the detection of ovarian carcinoma recurrences. In this regard, it is important that two patients who tested normal for CA125 showed little (<1 cm) (one patient) and no (one patient) extra-ovarian disease.

Only five of the ovarian tumors described in Table 1 showed mucinous differentiation. However, three of our five telomerase-negative tumors showed such differentiation. The difference in the rates of false-negativity between mucinous and nonmucinous tumors (three of five versus two of 37) was statistically significant ($P = .008$). In fact, the two patients with nonmucinous tumors who tested negative for telomerase had small (one patient) and microscopic (one patient) disease, whereas the three patients with mucinous tumors had extensive disease.

### Stability of Telomerase in Peritoneal Washings and Ascitic Fluids

The telomerase test would not be practical in clinical settings if it were dependent on special specimen storage or handling conditions. Therefore, we examined the stability of telomerase in unprocessed samples left at room temperature for up to 7 days. A representative experiment is shown in Fig. 1 and demonstrates that measurable telomerase activity can be detected in unprocessed samples left for up to 5 days at room temperature, even in samples with relatively low levels of activity such as the one shown in this figure.

### TRAP Assay Detection of Cancer Cells Admixed With an Excess of Telomerase-Negative Cells

One of the difficulties in detecting minimal residual cancer cells in treated patients comes from the fact that the test samples may contain large amounts of admixed normal cells. To verify that the presence of such cells does not interfere with the TRAP assay, we examined mixtures containing cells from various ovarian carcinoma cell lines expressing high levels of telomerase and increasing numbers of telomerase-negative diploid fibroblasts. The first lane of Fig. 2 shows the product of a TRAP assay performed on an extract derived from 50 HOC-7 cells (9) admixed with 100,000 fibroblasts. The characteristic ladder shown in Fig. 2 indicates that telomerase activity was readily detectable in this mixture. In contrast, an extract derived from 100,000 fi-
broblasts alone showed no detectable activity (Fig. 2, second lane). Similar experiments were performed with OVCAR-3, SKOV-3, and HEY cell lines (9). The results showed that the lowest number of cancer cells required to demonstrate telomerase activity in such admixtures varied between 10 and 50, depending on the level of telomerase expression in the individual cell lines. Thus, the TRAP assay is suitable for the detection of as few as 10–50 ovarian cancer cells, even in the presence of a large excess of admixed normal cells.

Specificity of the TRAP Assay

The potential clinical usefulness of the telomerase test for ovarian carcinoma depends not only on its sensitivity but also on its specificity. We were initially concerned that the test would, in fact, show little specificity for cancer cells in peritoneal fluid samples. The basis for this concern was that, although telomerase is not usually expressed in adult somatic cells, detectable levels of activity have been reported in activated B lymphocytes as well as in some stem cells. Therefore, we examined peritoneal washings from 29 patients who were undergoing surgery for benign gynecologic diseases and who had no cancer or history of cancer of any type. All 43 samples were examined cytologically as well as by the TRAP assay. Two samples tested positive for telomerase, whereas none of the samples was interpreted as positive by cytologic examination. Thus, the specificity of the TRAP assay was 95% compared with 100% for cytologic examination. This difference of 5% between the performance of the telomerase assay and cytologic examination was not statistically significant ($P = .50$). The two apparent false-positive results with the TRAP assay involved a sample of peritoneal washings from a patient with a benign ovarian teratoma and a sample of ascitic fluid from a patient with liver failure, which, although otherwise showing no evidence of cancer cells, contained a large amount of admixed inflammatory cells (not shown).

Discussion

Recent advances in our understanding of the molecular genetic changes associated with cancer have led to the development of novel and potentially powerful approaches for the clinical detection of this disease. Examples include the detection of allelic imbalances, microsatellite instabilities, and specific point mutations in cells, tissues, or fluids from various sources (13–17). The use of mutation-specific polymerase chain reaction primers for the detection of tumor-specific mutations, such as those found in oncogenes or tumor-suppressor genes, is another possibility (18). Although the sensitivity of measuring telomerase activity for cancer cell detection may vary with different cancers (19), the results of our experiments clearly attest to the potential of this approach in documenting the presence of such cells in concealed areas such as body cavities.

Several factors support the merit of this approach for the detection of ovarian carcinomas. First, telomerase is expressed in nearly 100% of these tumors (3). Second, the telomerase enzyme is exceptionally stable at room temperature, eliminating the need for special or rapid handling procedures at the time of specimen collection. Third, the high sensitivity of the TRAP assay ensures the detection of trace amounts of tumor cells in the presence of large excesses of normal cells. Fourth, peritoneal washings can easily be obtained from patients with ovarian carcinoma by use of minimally invasive techniques, such as laparoscopic procedures or lavage through long-term abdominal catheters, which are often placed in patients for the infusion of chemotherapeutic drugs. Finally, the fact that the TRAP assay measures an enzyme activity implies that it is dependent on the presence of intact cells expressing this enzyme. Thus, the TRAP assay may be a good indicator of viable as opposed to dead tumor cells. This is important because the medical treatment of advanced cancers undoubtedly leads to the release of large amounts of DNA from dying tumor cells. It is conceivable that released DNA fragments might remain in body cavities or in the circulation for long periods of time before they are cleared by the host, potentially complicating the interpretation of tests targeting tumor-specific molecular genetic changes in genomic DNA.

It is not clear why five patients, including two with substantial extra-ovarian disease ($>2$ cm), tested negative for telomerase in our study. Perhaps these false-negative results reflect suboptimal sampling, since our specimens were obtained from random peritoneal washings as opposed to multiple washings from various intra-abdominal sites. Another possibility is that specific tumor characteristics may be associated with false-negative results. For example, most of our patients with false-negative tests, including the two with extensive ($>2$ cm) extra-ovarian disease, had mucinous ovarian tumors. Perhaps the nature of those fluid samples, which were both very viscous, interfered with the telomerase assay. Thus, our proposed assay may not be applicable for the detection of ovarian tumors showing mucinous differentiation, particularly those with substantial amounts of mucin production. Additional studies are needed to define more clearly the determinants of false-negativity of telomerase testing in the above clinical setting to improve the reliability of this approach.

Additional studies are also needed to gain a better understanding of the various factors and conditions associated with false-positivity. For example, one of the two cancer-free control specimens that tested positive for telomerase was from a patient with an ovarian teratoma, suggesting that these benign tumors may express telomerase. The other telomerase-positive

Journal of the National Cancer Institute, Vol. 90, No. 3, February 4, 1998  REPORTS 241
control specimen contained a large number of inflammatory cells, implying that the assay may not be valid for cancer detection in such specimens. It should also be pointed out that the latter specimen was from a patient with advanced alcoholic liver disease. Although this patient otherwise showed no evidence of cancer, we cannot rule out the possibility that an occult malignant tumor was present, given the high rates of cancer associated with this clinical condition.

In conclusion, our results suggest that the presence of telomerase activity in abdominal fluids and washings from patients treated for advanced ovarian carcinoma may be a strong indicator of residual disease. Although the utility of this test in clinical settings remains to be proven, the idea that it could complement current conventional procedures, such as physical examination, radiographic studies, serum CA125 measurements, and even second-look laparotomies, merits further consideration. The hypothesis that telomerase testing of patients with no evidence of residual disease following adjuvant chemotherapy could help in the identification of individuals most likely to undergo disease recurrence is appealing, based on the low sensitivities of the current techniques. This approach may be particularly valuable for patients with disease confined to the upper abdomen, which is more likely to be missed by current techniques. Another possibility is that telomerase testing could be a useful component of patient follow-up protocols, since monitoring for telomerase activity in abdominal washings obtained from minimally invasive procedures may complement other conventional approaches and improve our ability to detect early disease recurrences.

References


Notes

Supported by Public Health Service grant R01 CA51167 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services; and by the Muggia Ovarian Cancer Research Fund. B. D. Duggan was supported by a 1995–96 Society for Gynecological Investigation–Mead Johnson Bristol-Myers Squibb research grant award.

We thank Dr. Gary Kanel for providing us with ascitic fluids from patients with liver disease.

Manuscript received July 25, 1997; revised October 30, 1997; accepted December 4, 1997.