Telomerase Assay for Differentiating between Malignancy-related and Nonmalignant Ascites

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ABSTRACT

The differential diagnosis between malignancy-related ascites (MRAs) and nonmalignant ascites (NMAs) has remained an essential problem in clinical practice. Our purpose was to determine the diagnostic value of ascitic fluid telomerase activity in discriminating these two categories compared with that of cytological examination. Twenty-five MRAs and 47 NMAs as the control group were enrolled in our study. In the MRA group, telomerase activity was detected in 13 of 16 (81.3%) cases of peritoneal carcinomatosis and in 6 of 9 (66.7%) cases of hepatocellular carcinoma (HCC)-associated ascites. Contrasting that, cytological examination was positive in only 9 of 16 (56.3%) and 1 of 9 (11.1%) cases, respectively. In the NMA group, telomerase-positive ascitic fluid samples were found in 2 of 47 (4.3%) cases, all belonging to subgroups that contained large numbers of lymphocytes in the ascites. In our study, the telomerase activity and cytological examination exhibited a sensitivity of 76% and 40% and a specificity of 95.7% and 100%, respectively. Regarding subgroups of MRAs, the telomerase activity and cytological examination demonstrated a sensitivity of 81.3% and 56.3%, respectively, in peritoneal carcinomatosis and a sensitivity of 66.7% and 11.1%, respectively, in HCC-associated ascites. In conclusion, telomerase activity is a more sensitive marker than cytological examination for differentiating between MRAs and NMAs. It may also serve as a useful indicator for detecting early i.p. metastasis in HCC-associated ascites.

INTRODUCTION

MRAs constitute a heterogeneous group of diseases with different pathophysiological mechanisms for ascites formation, accounting for 10% of all cases of ascites (1). In approximately two-thirds of patients with MRA, peritoneal carcinomatosis is associated with the implant of tumor cells in the peritoneal cavity (2). In general, tumors of any organ can metastasize to the peritoneum. Among these, gynecological tumors such as ovarian, endometrial, and cervical cancers are most common in females, whereas gastrointestinal cancers predominate in males (3). The remaining one-third of patients with MRA suffer from various conditions without tumor implantation in the peritoneum. The mechanisms of ascitic fluid formation in this subgroup are either portal hypertension-related ascites caused by massive liver metastasis, Budd-Chiari syndrome with or without inferior vena cava obstruction, as well as HCC superimposed on cirrhosis or chylous ascites caused by lymph node obstruction in lympho-proliferative disorders (2).

The differential diagnosis between MRAs and NMAs has remained a clinical challenge. Although cytological examination of ascitic fluid is considered the gold standard in terms of diagnostic specificity, its sensitivity in detecting MRA is low, ranging between 40% and 60% (4, 5). Various laboratory parameters such as total protein, lactate dehydrogenase, fibronectin, and cholesterol from ascitic fluid, as well as the SAAG, have been evaluated, but none of them are satisfactory as a single diagnostic test (6–11). Likewise, several tumor markers such as carcinoembryonic antigen, cancer antigen 125 (CA 125), and carbohydrate antigen 19-9 (CA 19-9) have also been evaluated, but discrepancies among these tests still persist (11–13). Therefore, a more reliable test with higher sensitivity and specificity to discriminate between MRA and NMA is required.

Telomerase represents a ribonucleoprotein complex that synthesizes a specific repetitive nucleotide sequence (TTAGGG), onto the ends of telomeres, preventing their shortening (14). The activation of this enzyme is thought to be responsible for preventing cellular senescence and developing cancer (15, 16). Telomerase activity has not been demonstrated in most somatic cells, but it is usually expressed in a large number of cancer cell lines (17). Because this enzyme activity is unique to immortal cells, it can be an important marker for diagnosing a wide variety of cancers.

Recently, a PCR-based telomerase assay, the TRAP assay, has been applied to detect the malignant phenotype in a variety of human carcinomas (for example, ovary, liver, colon cervix, and gastric tumors; Refs. 18–22). At present, numerous data...
suggest that telomerase may be the most widely expressed and specific tumor marker known to date (23). It has also been shown that the TRAP assay was sensitive enough to detect the presence of telomerase activity among as few as 1–10 immortal cancer cells and 0.01% positive cells in a mixed population (17). Thus, using the TRAP assay to detect the telomerase activity in the cells obtained from ascitic fluid, as shown in this study, could provide a potentially useful noninvasive method to differentiate MRA from NMA.

MATERIALS AND METHODS
Seventy-two patients with ascites in Chulalongkorn Hospital were enrolled prospectively into this study. These patients were divided into groups on the basis of the cause of ascites.

Group 1 consisted of 25 patients with MRA. They were further divided into two subgroups.

- Group 1A consisted of 16 patients with peritoneal carcinomatosis: 4 with liver metastasis and 14 without. Diagnosis was based on histological evidence with tissue specimens obtained from exploratory laparotomy or laparoscopy in patients with ovarian \( (n = 7) \), colon \( (n = 2) \), gastric \( (n = 2) \), and pancreatic cancer \( (n = 1) \). No primary site was identified in the remaining four cases.

- Group 1B consisted of nine patients with HCC superimposed on cirrhosis. The diagnosis of HCC was based on liver tumor features detected by ultrasound and confirmed by histology and/or serum alpha-fetoprotein levels above 400 IU/ml. In all patients, the finding of ascites was concomitant with the diagnosis of HCC.

Group 2 consisted of 47 patients with NMA. They were further divided into three subgroups.

- Group 2A comprised 30 patients with sterile uncomplicated cirrhotic ascites. Diagnosis of liver cirrhosis was confirmed by clinical features (for example, presence of esophageal varices at endoscopy and typical characteristics shown by ultrasonography). In each patient, the serum alpha-fetoprotein level was <10 IU/ml and the ultrasound showed no evidence of malignancy.

- Group 2B consisted of 10 patients with cirrhosis and SBP. In all patients, the bacterial cultures were positive and the PMN count in ascites exceeded 250/mm\(^3\).

- Group 2C consisted of seven patients with tuberculous peritonitis diagnosed by the presence of caseating granulomas on peritoneal biopsy obtained from laparoscopy.

Specimens of ascitic fluid were obtained by percutaneous paracentesis in cirrhotic patients with or without HCC and obtained during laparoscopy or exploratory laparotomy in cases undergoing those procedures. All fluids obtained in the course of surgery were collected immediately after the initial surgical incision to avoid contamination of blood-cell components. The fluids were then sent for routine studies, such as cell count and differential, Gram’s stained smear and culture, total protein, and albumin. Blood samples for serum albumin were also taken at the same time as the above measurements to calculate the SAAG. Apart from routine examinations, the ascitic fluid specimens were divided into two parts. The first specimen (100 ml of fluid) was submitted to cytological examination, and the second specimen (20 ml of fluid) was prepared for the TRAP assay. Cytological examination was performed by a cytopathologist (P. S.) as part of the research protocol. Data acquired from each protocol, TRAP assay, and cytological examination were collected in a double-blind fashion until further analysis.

After this initial examination, when discrepancies existed between cytological examination and the TRAP assay, the cytological specimens were reevaluated by the same cytopathologist.

TRAP Assay. TRAP was performed as described previously (17, 24). Briefly, each sample was first centrifuged and washed in 500 \( \mu \)l of ice-cold PBS, then homogenized in 20–200 \( \mu \)l of ice-cold 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate lysis buffer according to the sample size with a manual homogenizer. The collagenase-treated specimens were washed with ice-cold PBS and treated with lysis buffer without homogenization. After a 30-min incubation on ice, the lysate was centrifuged at 14,000 \( \times \) g for 30 min at 4°C. The supernatant was divided into aliquots, flash-frozen in liquid nitrogen, and stored at −80°C until further analysis. An aliquot of the extract containing 6 \( \mu \)g of protein was used for each TRAP assay, EBV-transformed human lymphocytes (ATCC B958) were used as positive controls.

An aliquot of 1 \( \mu \)g of TS substrate primer was end-labeled in a 10-\( \mu \)l reaction mixture with 10 \( \mu \)Ci \([\gamma-32P]\)dATP (3000 Ci/mmol). The PCR-based assay was carried out in a 25-\( \mu \)l reaction mixture containing 6 \( \mu \)g of protein from the lysate, 1XTRAP buffer (17), 50 mM dNTPs, 0.1 \( \mu \)g of labeled TS primer, 0.005 \( \mu \)mol TSNT internal control, 0.005 \( \mu \)g of NT primer, 2 units of Taq polymerase, and DEPC \( \text{H}_2\text{O} \) in a 0.5-ml tube containing 0.1 \( \mu \)g of ACX sealed at the bottom by a wax barrier.

After a 10-min incubation at 23°C to allow telomerase-mediated extension of the TS primer, the reaction mixture was subjected to 31 PCR cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. Aliquots (5 \( \mu \)l) of the PCR products were analyzed on an 8% non-denaturing polyacrylamide gel. The gel was subsequently exposed to a phosphor screen, and the bands were visualized on a PhosphorImager using Image Quant software (Molecular Dynamics, Sunnyvale, CA). Duplicate assays were performed on all samples with RNase pretreatment at a final concentration of 0.05 mg/ml for 10 min at room temperature.

The samples exhibiting negative internal control, interpreted as presenting a PCR inhibitor, were subjected to two-step TRAP.4 The first part consisted of TS primer extension in a 25-\( \mu \)l reaction mixture containing 1XTRAP buffer, 50 mM dNTPs, 0.1 \( \mu \)g of TS, and DEPC \( \text{H}_2\text{O} \). The reaction mixture was incubated in the thermocycler at 23°C for 1 min. The product was then subjected to standard phenol-chloroform DNA extraction and ethanol precipitation. The precipitate was dissolved in 25\( \mu \)l of DEPC \( \text{H}_2\text{O} \) and amplified in the second round. The second round reaction mixture was identical to the described TRAP, but omitting the incubation at 23°C for 15 min. The amplification cycle was the same as in the original protocol.

All negative TRAP results were tested for the possible presence of a telomerase inhibitor. Lysates from 10\(^7\) cells of the

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4 J. W. Shay, personal communication.
B958 lymphocyte cell line were added, and the mixed samples were subjected to either one- or two-step, if a PCR inhibitor was present, TRAP assay.

**Statistical Analysis.** Data were presented as mean ± SD. The ANOVA and Student’s t test were used to assess the statistical significance of the difference between the groups. A P ≤ 0.05 was considered statistically significant. Sensitivity, specificity, positive and negative predictive values, and diagnostic accuracy were calculated in accordance with standard methods.

**RESULTS**

Seventy-two patients (35 men and 37 women; ages 23–82 years, with a mean of 54.2 ± 14.9 years) were enrolled into our study. As shown in Table 1, the ascitic fluid WBC count in groups 1A and 2C comprised predominantly lymphocytes, whereas in group 2B it consisted mainly of PMN cells. The mean ascitic fluid protein contents in groups 1A and 2C were significantly higher than in groups 1B, 2A, and 2B. Because the total protein content was above 2.5 g/dl, the ascitic fluid in groups 1A and 2C should be classified as exudative ascites. On the other hand, the mean SAAG in groups 1A and 2C were significantly lower than in groups 1B, 2A, and 2B. These results confirmed that neither the total protein content nor SAAG could be considered sensitive and specific parameters for the diagnosis of MRA.

**Telomerase Activity in Ascitic Fluid.** Negative examples with absent TRAP, but with the internal control signal present, and positive telomerase activity obtained from ascitic cells are shown in Fig. 1. Because PCR and telomerase inhibitors could increase the number of false negative samples, we performed additional experiments to investigate these two possibilities. The presence of a PCR inhibitor was detected by the absence of both TRAP and the internal control signal and was abolished by a two-step experiment. Among 72 cases, 19 cases showed the inhibitor. After the two-step assay, telomerase activity was recovered for six cases (31.6%). This proportion is comparable with samples without PCR inhibitor (28%) and the total number of cases (29%). The presence of a telomerase inhibitor was determined by the addition of a known positive extract to negative samples. No absence of telomerase activity was observed.

In our study, 13 of 16 (81.3%) and 6 of 9 (66.7%) samples in groups 1A and 1B, respectively, were positive for telomerase activity. Overall, the telomerase assay performed in both subgroups, which represented MRA in this study, yielded positive results in 19 of 25 samples (76%). In the present study, all samples yielding negative findings for telomerase activity were also negative by fluid cytology (Table 2).

NMA representing our control group were also examined for the possible presence of telomerase activity. There were 2 of 47 (4.3%) cases positive for the assay (one cirrhotic ascite and one tuberculous peritonitis). The positive rate in sterile (group 2A) and infected cirrhotic ascites (group 2B) amounted to 0% (0 of 30) and 10% (1 of 10), respectively, whereas the positive rate in tuberculous peritonitis (group 2C) was 14.3% (1 of 7).

The results showed that positive results of the TRAP assay were highly correlated with MRA, particularly among peritoneal carcinomatosis. In our study, the overall sensitivity and specificity of the assay were 76% and 95.7%, respectively. The positive and negative predictive values amounted to 90.5% and 88.2%, respectively, and the overall accuracy was 88.9%. On comparing group 1A with the controls (group 2), sensitivity and accuracy were increased up to 81.3% and 92.1%, respectively. On comparison between groups with exudative, lymphocytic ascites profiles (1A and 2C), the sensitivity of the TRAP assay was 81.3%, whereas specificity and accuracy were 88.6% and 92.1%, respectively. Likewise, in cirrhotic patients with or without HCC (groups 1B, 2A ,and 2B), the sensitivity of the assay was 66.7%, whereas the specificity and accuracy were as high as 97.5% and 91.8%, respectively.

**Cytological Examination of Ascitic Fluid.** Ascitic samples from groups 1A and 1B showed positive results of the cytological examination in 9 of 16 (56.3%) and 1 of 9 (11.1%) samples, respectively. In our study, none of the samples obtained from group 2 showed positive fluid cytology (Table 2). The overall sensitivity and specificity of the cytological examination were 40% and 100%, respectively, and the diagnostic accuracy was 79.2%.

**DISCUSSION**

In general, the diagnosis of most forms of cancer almost always is confirmed by pathological examination, either directly from tissue biopsy or through easily obtainable body fluids such as ascitic fluid. Telomerase is a reverse transcriptase that is always present in cancer cells and is absent in normal cells. Thus, it is considered to be a sensitive and specific marker for cancer diagnosis. Recently, telomerase activity has been detected in a wide variety of human cancers. The presence of telomerase activity was confirmed by PCR and TRAP, but the activity was abolished by the presence of MRA inhibitors. The results showed that positive results of the TRAP assay were highly correlated with MRA, particularly among peritoneal carcinomatosis. In our study, the overall sensitivity and specificity of the assay were 76% and 95.7%, respectively. The positive and negative predictive values amounted to 90.5% and 88.2%, respectively, and the overall accuracy was 88.9%. On comparing group 1A with the controls (group 2), sensitivity and accuracy were increased up to 81.3% and 92.1%, respectively. On comparison between groups with exudative, lymphocytic ascites profiles (1A and 2C), the sensitivity of the TRAP assay was 81.3%, whereas specificity and accuracy were 88.6% and 92.1%, respectively. Likewise, in cirrhotic patients with or without HCC (groups 1B, 2A ,and 2B), the sensitivity of the assay was 66.7%, whereas the specificity and accuracy were as high as 97.5% and 91.8%, respectively.
as blood, urine, or ascites. As mentioned above, cytology evaluation of ascitic fluid yields a high diagnostic specificity but a low sensitivity in differentiating between MRA and NMA. This lack of sensitivity may be due to inadequate sample collection obtained from abdominal paracentesis or to a low number of tumor cells shed into the fluid specimens, particularly in those without peritoneal carcinomatosis. Moreover, false-negative results could be caused by the difficulty in interpreting the morphological cytology among neoplastic and atypical inflammatory cells (26, 27).

Our study showed that the overall sensitivity of the TRAP assay was superior to that of cytology examination (76% and 40%, respectively), whereas the specificity of the two tests was comparable. These results support that the TRAP assay provides a more accurate means than cytology for the diagnosis of MRA. However, the overall sensitivity of the TRAP assay in our study was slightly lower than that previously reported in ovarian cancers (76% and 88%, respectively; Ref. 28). This difference could be explained by the fact that our population included heterogeneous subgroups of MRA. If HCC-associated ascites were excluded, the diagnostic sensitivity would be as high as 81.3% and even higher among ovarian cancers (85.7%). In recent studies, although an average incidence of telomerase activation among various malignant tumors was as high as 85%, a wide variability of telomerase expression was also observed in different cancer groups (23, 29). Among these, kidney, ovarian, and breast cancers showed the highest mean values of telomerase activity assessed by a quantitative method (30).

The high specificity of telomerase activity by the TRAP assay in this study was clearly demonstrated among patients with NMA. Although telomerase activity was also detected in two samples, one from uncomplicated cirrhotic ascites and the second one from tuberculous peritonitis, the number of telomerase-positive samples was low compared with that of MRA. Our data, therefore, confirmed previous reports regarding the diagnostic accuracy of the TRAP assay in effusions of body cavities (28, 31). One possible explanation for the false positive result in the cirrhotic patient is that minute HCC often coexists with advanced cirrhosis and is not easily diagnosed by clinical features (32). Therefore, we could not completely exclude the possibility of an undetected tumor in the patient otherwise showing no evidence of cancer.

Another possible explanation is that ascitic fluid often contains large numbers of blood-cell components and the contaminating activated lymphocytes may be responsible for weak, but detectable, telomerase activity. This observation has been mentioned previously in that certain populations of normal blood-cell components could express low levels of telomerase activity (33–35). Interestingly, the two telomerase-positive cases in NMA in our study all belonged to subgroups that contained large numbers of lymphocytes, particularly in tuberculous ascites. Because ascitic fluid in tuberculosis is almost always exudative (protein content above 2.5 g/dl) with low SAAG (<1.1 g/dl) and consists predominantly of lymphocytes (36), it is sometimes difficult to differentiate it from that of peritoneal carcinomatosis, as shown in Table 1. Thus, in countries in which tuberculosis is endemic, this possible false positive telomerase activity should not be ignored.

Ascites among patients with HCC is a poorly characterized subgroup of MRA (2, 37). Clinically, HCC usually develops as a complication of long-standing cirrhosis resulting from either chronic viral hepatitis or from heavy alcohol consumption (38). Therefore, not only the underlying liver disease, but also the tumor growth and spread, contribute to ascites formation by

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**Table 2** Telomerase activity and cytology in MRA and NMA

<table>
<thead>
<tr>
<th>Group</th>
<th>Underlying diseases</th>
<th>No. TRAP positive</th>
<th>No. cytology positive</th>
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<tr>
<td>MRA (25)</td>
<td>Ovarian cancer (7)</td>
<td>6</td>
<td>5</td>
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<td></td>
<td>Colon cancer (2)</td>
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<td>Gastric cancer (2)</td>
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<tr>
<td></td>
<td>Unknown primary (4)</td>
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<td>2</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>13 (81.3%)</td>
<td>9 (56.3%)</td>
</tr>
<tr>
<td>1B (9)</td>
<td>Cirrhosis with HCC</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Total (%)</td>
<td>6 (66.7%)</td>
<td>1 (11.1%)</td>
</tr>
<tr>
<td>Total (1A and 1B)</td>
<td>19 (76.0%)</td>
<td>10 (40%)</td>
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<tr>
<td>NMA (47)</td>
<td>Cirrhosis without SBP (30)</td>
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<td>0</td>
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<tr>
<td></td>
<td>Cirrhosis with SBP (10)</td>
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<td>0</td>
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<td></td>
<td>Tuberculosis (7)</td>
<td>1</td>
<td>0</td>
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<tr>
<td></td>
<td>Total</td>
<td>2 (4.3%)</td>
<td>0 (0%)</td>
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either increasing the portal pressure or, less frequently, by lymphatic obstruction or infiltration of the peritoneum (37). Nonetheless, it is important, for both clinical staging and treatment, to distinguish between ascites arising from cirrhosis and ascites as a consequence of HCC, per se.

The mechanism of peritoneal seeding in HCC is not clear. One pathway of peritoneal seeding is thought to stem from a rupture of exophytic HCC into the peritoneal cavity and subsequent seeding of metastatic deposits (39). Although distant dissemination in the Douglas’ pouch occurs in a certain percentage of cases, generalized implants involving the entire peritoneum, or the so-called peritoneal carcinomatosis, which occurs frequently in pancreatic and gastric cancer, is rarely present (40). From this observation, the rarity of malignant cells detected in ascitic fluid could be explained. Hence, the yield of cytology examination in HCC-associated ascites is not satisfactory, with positive results amounting to ~0–12% in most series (2, 4, 32, 37).

Our results clearly showed a high percentage of positive TRAP assay in HCC-related ascites compared with that obtained by cytology examination (66.7% and 11.1%, respectively). Furthermore, the TRAP assay discriminates between HCC and cirrhosis with a satisfactory diagnostic accuracy (91.8%). The fact that telomerase was present in ascites, despite negative cytology results, suggested a minimal amount of neoplastic cells shedding into the fluid. Thus, it probably indicated either an early stage of i.p. spread of the superficial HCC or represented a leakage from minute ruptured HCC into the peritoneal cavity. These data suggest that the TRAP assay in ascites could provide a useful diagnostic marker for detecting early i.p. metastasis, as well as minute spontaneously ruptured HCC. Moreover, yet to be addressed is whether telomerase activity in ascitic fluid could be useful as a prognostic indicator for the clinical outcome in these patients. In this regard, it is noteworthy from previous studies that telomerase expression correlates well with the outcome in these patients. In this regard, it is noteworthy from previous studies that telomerase expression correlates well with the outcome in these patients (19, 41).

In conclusion, our data suggest that the presence of telomerase activity in ascitic fluid is a valuable indicator for the diagnosis of MRA, particularly among patients with peritoneal carcinomatosis. In addition, the telomerase assay may improve the early detection of i.p. metastasis among patients with HCC-associated ascites. Further improvements in the telomerase detection techniques, as well as additional clinical studies, are still required to assess the validity of the assay as a diagnostic or prognostic marker in these patients.

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