Advantages of assaying telomerase activity in ascites for diagnosis of digestive tract malignancies

Chung-Pin Li, Tze-Sing Huang, Yee Chao, Full-Young Chang, Jacqueline Whang-Peng, Shou-Dong Lee

INTRODUCTION
Ascites is a common complication of malignancy. Carcinoma of any organ can metastasize to the peritoneum. Gastric, colon, pancreatic, ovarian, breast, and lung carcinomas, as well as lymphoma, are the most common tumors associated with malignant ascites[1]. Hepatocellular carcinoma (HCC) is the leading cause of cancer mortality in Asia[2]. Ascites is also commonly seen in HCC patients, especially in terminal stage or when complicated with liver cirrhosis (LC). The pathophysiological mechanisms for the ascites production in LC are quite distinct from those for malignancy-related ascites[3]. The conventional diagnostic methods are sometimes inefficient and unsatisfactory to diagnose ascites as malignancy-related. For example, malignant ascites could be diagnosed by cytological examination of the ascitic fluids in only 50-70% of patients with ascites[4].

Telomeres (special DNA structures that contain TTAGGG repeats at the ends of all eukaryotic chromosomes) have important functions in protecting genomic DNA from degradation and deleterious recombination events[4]. Telomerase, an RNA-dependent DNA polymerase, can maintain the telomeric length by acting as a reverse transcriptase[5]. In humans, tumor cells escape programmed cell senescence through reactivation of telomerase[6]. These immortalized cells can compensate for telomeric shortening at each cell division, leading to progressive neoplastic evolution[6,7]. Telomerase re-expression was found in 85% of human malignant tumors[8,9]. The use of telomerase assay for cancer diagnosis might be possible because telomerase is thought to be re-expressed in malignant lesions but rarely in normal somatic cells[10,11]. Some studies also suggested that the telomerase activity of ascites cells could be a sensitive marker for differentiating malignancy-related ascites from nonmalignant ascites[11,12]. However, these studies included only small numbers of gastrointestinal tract cancer and HCC patients.

This study was to assess the diagnostic value of using telomerase activity assay to distinguish digestive tract cancer-derived ascites from benign ascites.

MATERIALS AND METHODS
Patients
Ninety-five patients with ascites undergoing a therapeutic or diagnostic paracentesis in Taipei Veterans General Hospital were enrolled prospectively into this study. These patients were divided into three groups on the basis of the cause of the ascites. HCC Group consisted of 40 patients with HCC. The diagnoses were made by imaging studies (abdominal sonography and/or computed tomography and/or magnetic resonance imaging) and serum α-fetoprotein levels above 400 IU/mL in 34 patients, and were confirmed by liver biopsy in the remaining 6 patients.

CA Group consisted of 31 patients with non-HCC gastrointestinal carcinoma, including 10 gastric, 8 colon, 10 pancreatic cancers, and 3 cholangiocarcinomas (19 with liver metastasis and 12 without). All diagnoses were histologically confirmed.

LC Group comprised 24 patients with sterile uncomplicated cirrhotic ascites. Liver cirrhosis was diagnosed by typical clinical findings (splenomegaly, ascites and/or esophageal varices, splenomegaly, and ascitic protein concentration, but to white blood count (58%) and 1 HCC patient (2.5%), respectively. The positive correlation between telomerase activity and the C-reactive protein level was significant (r = 0.31, P = 0.005), neutrophil count (r = 0.29, P = 0.005), and the C-reactive protein level (r = 0.29, P = 0.018). When the results of both cytological examination and telomerase assay were considered together, the sensitivity increased to 77% for CA patients, 25% for HCC patients, and 48% for all 71 gastrointestinal cancer patients.

CONCLUSION: Combining cytological examination of ascites with telomerase activity assay significantly improves the differential diagnosis between malignant and non-malignant ascites.

Li CP, Huang TS, Chao Y, Chang FY, Whang-Peng J, Lee SD. Advantages of assaying telomerase activity in ascites for diagnosis of digestive tract malignancies. World J Gastroenterol 2004; 10(17): 2468-2471
http://www.wjgnet.com/1007-9327/10/2468.asp
varices), imaging studies (abdominal sonography and/or computed tomography and/or magnetic resonance imaging) and laboratory findings in 20 patients, and was confirmed by a liver biopsy in the remaining 4 patients. The serum α-fetoprotein level was <20 IU/mL, and an ultrasound showed no evidence of malignancy in each patient.

Aritic fluids were subjected to routine assays, such as cell count and categorization, Gram’s stain and culture, total proteins, albumin, and cytological examination. Blood samples for serum albumin determination were also taken at the same time as the above measurements to calculate the serum-ascites albumin gradient (SAAG). The ascites was also centrifuged and the sediment was detected with the Telomeric Repeat Amplification Protocol (TRAP). The samples were collected in heparinized containers and centrifuged at 2 000 r/min for 10 min at 4 °C. The supernatant was decanted. Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and were stored in a 1.5-mL tube at -80 °C until assay. For bloody specimens, excessive RBCs were removed with Ficoll-Hypaque density-gradient centrifugation[14]. All samples were coded so that technicians running the assays were blinded as to the source of the samples.

Telomeric repeat amplification protocol (TRAP)

The telomerase activity was assayed according to the protocol described previously[15]. Cell lysates were prepared in 200 μL of lysis buffer consisting of 10 mmol/L Tris-HCl (pH 7.5), 5 g/L CHAPS, 1 mmol/L MgCl2, 1 mmol/L EGTA, 100 mL/L glycerol, 0.1 mmol/L benzamidine, and 5 mmol/L β-mercaptoethanol. Aliquots of extracts were treated with or without RNase A (Sigma, St. Louis, MO) in a final concentration of 0.05 mg/mL for 30 min at 37 °C. Then, each TRAP reaction was performed at 30 °C for 30 min in 50 μL of reaction mixture containing 200 ng of cell lysates, 20 mmol/L Tris-HCl (pH 8.3), 0.5 g/L Tween-20, 1.5 mmol/L MgCl2, 63 mmol/L KCl, 1 mmol/LE GTA, 250 μmol/L dNTPs, Primer Mix (Intergen Co., Purchase, NY), and 2 units of Taq polymerase (Roche Molecular Biochemicals, Indianapolis, IN), and followed by 33 cycles of PCR: at 94 °C for 0.5 min and 59 °C for 0.5 min. One half of each reaction mixture was resolved in a 12.5 g/L polyacrylamide gel, and after staining with SYBR® Green (Molecular Probes, Eugene, OR), the image of the telomeric repeat pattern was printed and quantified within a range from 50 to 100 base pairs by Alpha-InnoTech IS500 Digital Imaging System (Avery Dennison, CA). All samples were run in duplicate and the reproducibility was confirmed. All extracts that produced 6-base-pair ladders were tested for sensitivity to RNase A and specificities were confirmed by Southern blotting. Representative examples of the results are shown in Figure 1.

Table 1 Clinical characteristics and ascitic fluid analyses

|                   | CA       | HCC      | LC       |
|-------------------|----------|----------|----------|
| Number of patients| 31       | 40       | 24       |
| Age (yr)          | 65±2     | 65±2     | 67±2     |
| Sex: Male/Female  | 21/10    | 35/5     | 18/6     |
| Protein (g/dL)    | 2.9±0.2  | 1.5±0.1  | 1.5±0.2  |
| SAAG (g/dL)       | 1.1±0.1  | 2.0±0.2  | 2.0±0.2  |
| WBC (cells/mm³)   | 481±107  | 847±590  | 149±27   |
| RBC (cells/mm³)   | 13 093±7 975 | 192 633±104 982 | 3 730±1 515 |
| PMN (cells/mm³)   | 177±78   | 698±550  | 20±8     |
| Lymphocytes (cells/mm³)  | 305±77  | 149±46  | 128±26   |

Data are expressed as mean±SE. CA: non-HCC gastrointestinal cancer, HCC: hepatocellular carcinoma, LC: liver cirrhosis, SAAG: serum-ascites albumin gradient, WBC: white blood count, PMN: polymorphonuclear cells. *P<0.05, †P<0.01, ‡P<0.001 if compared with CA patients.
**Table 2** Comparison of telomerase activity assay and cytological examination (n, %)

| Group (n) | Underlying diseases (n) | Positive telomerase | Positive cytology |
|-----------|-------------------------|---------------------|------------------|
| CA (31)   | Gastric cancer (10)     | 16 (52)             | 18 (58)          |
|           | Colon cancer (8)        | 5 (50)              | 6 (60)           |
|           | Pancreatic cancer (10)  | 3 (38)              | 4 (50)           |
|           | Cholangiocarcinoma (3)  | 7 (70)              | 8 (80)           |
| HCC (40)  | 1 (33)                  | 0 (0)               |                  |
| LC (24)   | 10 (25)                 | 1 (2.5)             |                  |
|           | 1 (4)                   | 0 (0)               |                  |

CA: non-HCC gastrointestinal carcinoma, HCC: hepatocellular carcinoma, LC: liver cirrhosis.

Figure 1 Representative results of ascitic telomerase activity assay. Lane P: positive control, Lane N: heat-inactivated sample, TSR8, quantitation control, CA: non-HCC gastrointestinal carcinoma, HCC: hepatocellular carcinoma, LC: liver cirrhosis.

It was noted that telomerase activity was higher in the ascites of CA patients than in the ascites of HCC and LC patients (CA: 22.9±5.8, HCC: 6.7±2.5, LC: 1.3±1.3, P = 0.001, Figure 2). In addition, as shown in Table 3, the telomerase activity was not related to patients’ age, gender, serum albumin and ascitic protein amount, but to patients’ white blood count (r = 0.31, P = 0.002), blood neutrophil count (r = 0.29, P = 0.005), and C-reactive protein level (r = 0.29, P = 0.018).

**Figure 2** Individual and mean values of telomerase activities in CA, HCC and LC ascites.

When the results of both cytological examination and telomerase assay were combined together, the sensitivity increased to 77% (24/31) for CA patients, 25% (10/40) for HCC patients, and 48% (34/71) for the 71 gastrointestinal cancer patients. While cytological examination alone had a sensitivity of 27% (19/71) and telomerase assay alone had a sensitivity of 37% (26/71).

**Table 3** Correlation between ascitic telomerase activity and clinical parameters

| Parameter            | r   | P  |
|----------------------|-----|----|
| Age (yr)             | -0.05 | 0.65 |
| Sex: Male/Female     | -0.18 | 0.09 |
| Serum albumin        | -0.03 | 0.11 |
| Ascites protein      | 0.08  | 0.44 |
| Blood WBC            | 0.31  | 0.002 |
| Blood neutrophil     | 0.29  | 0.005 |
| Serum C-reactive protein | 0.29 | 0.018 |

WBC: white blood count.

**DISCUSSION**

Diagnosis of cancer primarily depends on pathological examination. Ascites is a common complication of malignant neoplasms. The conventional cytological assessment of ascitic fluid can provide highly specific and accurate diagnoses of malignancies, but its unsatisfactory low sensitivity does not allow pathologists to early differentiate malignant ascites from non-malignant ascites[16,17]. Some studies have suggested that assay of telomerase activity in ascites might be an alternative method to improve the diagnosis[11,12]. However, only small numbers of patients with gastrointestinal tract cancers and HCC were included in those studies. The present study is the first one evaluating the value of telomerase activity assay in differential diagnosis of malignant and nonmalignant ascites of gastrointestinal cancers.

In our study, the sensitivity of telomerase activity assay of ascites from CA patients was 52%, comparable to that (58%) by cytological examination. This result is consistent with a previous report[11]. However, our TRAP sensitivity seemed to be less than that reported in another paper about ovarian cancers[17]. Although the average incidence of abnormal telomerase activity occurred in as high as 85% of human cancer patients, the incidence varied with different cancer types[8,18]. Among these, kidney, ovarian, and breast cancers showed the highest mean values of quantitatively assessed telomerase activities[19].

Ascites is commonly seen in HCC patients, especially in decompensated stages. Concomitant cirrhosis was present in more than 97% of HCC patients[20]. Not only the tumor growth, but also the underlying liver cirrhosis, led to ascites[20,21]. In analysis of our 24 LC and 40 HCC patients, only one HCC patient had a positive cytological diagnosis. This agrees with previously published data showing that malignant cells detected in ascites occurred in only 0-12% of the HCC patients[20-24], suggesting cytological examination is not an efficient tool for distinguishing HCC ascites from non-HCC LC ascites. Assay of telomerase activity (which was positive in 25% of HCC ascites samples were positive) is obviously better than the conventional cytological assessment.

Moreover, we speculated combining the results of both cytological examination and telomerase activity assay might improve diagnosis. We were able to identify malignancy in 77% of CA patients and 25% of HCC patients. For all 71 gastrointestinal cancer patients, the sensitivity of diagnosis increased to 48% (by the combination of both techniques) from 27% (by the cytological examination alone). Our study results thus indicate that TRAP assay is a useful tool for detecting cancer cells in ascites from gastrointestinal cancer patients.

Noteworthily, ascitic telomerase activity of gastrointestinal cancers is correlated with the increase in blood leukocytes and C-reactive protein. Leukocytosis and C-reactive protein are major markers of inflammation and cancer[25,26]. The source of
telomerase activity in ascitic cells could be cancer cells shed into the ascitic fluid or ascitic fluid leukocytes that were upregulated by cancer-related cytokines\(^{27-35}\). Whatever the mechanism(s), assay of telomerase activity in ascitic cells may provide an adjunct to cytological examination in the diagnosis of digestive tract cancers.

**REFERENCES**

1. Parsons SL, Watson SA, Steele RJ. Malignant ascites. *Br J Surg* 1996; 83: 6-14
2. Tang ZY, Liu YF, Zhang JF, Zhang SX, Li DF, Yang JJ. Expression and function of interleukin-8 in human hepatocellular carcinoma. *Cancer* 1997; 93: 677-682

3. Yuasa S, Itoshima T, Nagashima H. Clinical studies of hepatozellular carcinoma with liver cirrhosis and ascites. *Acta Med Okayama* 1984; 38: 291-299

4. Runyon BA, Hoefs JC, Morgan TR. Ascitic fluid analysis in malignancy-related ascites. *Hepatology* 1988; 8: 1104-1109

5. Toniatti C, Arcone R, Majello B, Ganter U, Arpaia G, Ciliberto G. Regulation of the human C-reactive protein gene, a major marker of inflammation and cancer. *Mol Biol Med* 1990; 7: 199-212

6. Mahmoud FA, Rivera NI. The role of C-reactive protein as a prognostic indicator in advanced cancer. *Curr Opin Oncol* 2002; 4: 250-255

7. Akiba J, Yano H, Ogasawara S, Higaki K, Kojiro M. Expression and function of interleukin-8 in human hepatocellular carcinoma. *Int J Oncol* 2001; 18: 257-264

8. Chau GY, Wu CW, Lui WY, Chang TJ, Kao HL, Wu LH, King KL, Loong CC, Hsia CY, Chi CW. Serum interleukin-10 but not interleukin-6 is related to clinical outcome in patients with resectable hepatocellular carcinoma. *Ann Surg* 2000; 231: 552-558

9. Price JA, Kovach SJ, Johnson T, Koniaris LG, Cahill PA, Sitzmann JV, McKillop IH. Insulin-like growth factor I is a comitogen for hepatocyte growth factor in a rat model of hepatocellular carcinoma. *Hepatology* 2002; 36: 1089-1097

10. Su Q, Liu YF, Zhang JF, Zhang SX, Li DF, Yang JJ. Expression of insulin-like growth factor II in hepatitis B, cirrhosis and hepatocellular carcinoma: its relationship with hepatitis B virus antigen expression. *Hepatology* 1994; 20(4 Pt 1): 788-799

11. Akiyama M, Hideshima T, Hayashi T, Tai YT, Mitsiades CS, Mitsiades N, Chauhan D, Richardson P, Munshi NC, Anderson KC. Cytokines modulate telomerase activity in a human multiple myeloma cell line. *Cancer Res* 2002; 62: 3876-3882

12. Wilson J, Balkwill F. The role of cytokines in the epithelial cancer microenvironment. *Semin Cancer Biol* 2002; 12: 113-120

13. Counter CM, Gupta J, Harley CB, Leber B, Bacchetti S. Telomerase activity in normal leukocytes and in hematologic malignancies. *Blood* 1995; 85: 2315-2320

14. Hiyama K, Hira i, Kyoiizumi S, Akiyama M, Hiyama E, Piatyszek MA, Shay JW, Ishioka S, Yamakido M. Activation of telomerase in human lymphocytes and hematopoietic progenitor cells. *Immuno*ol 1995; 155: 3711-3715

15. Weng NP, Levine BL, June CH, Hodes RJ. Regulated expression of telomerase activity in human T lymphocyte development and activation. *J Exp Med* 1996; 183: 2471-2479

Edited by Zhu LH and Xu FM