Sensitive and Specific Detection of Circulating Cancer Cells in Patients with Hepatocellular Carcinoma; Detection of Human Telomerase Reverse Transcriptase Messenger RNA after Immunomagnetic Separation

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ABSTRACT

Purpose: We evaluated whether detection of human telomerase reverse transcriptase (hTERT) mRNA after immunomagnetic separation is useful to detect circulating cancer (CC) cells.

Experimental Design: Two ml of peripheral blood were collected from 55 cases with hepatocellular carcinoma (HCC), 20 cases with chronic liver diseases devoid of cancer, and 20 healthy volunteers. Then 1500 and 500 µl were subjected to immunomagnetic separations using Ber-EP4 and anti-CD45 antibodies, harvested and supernatant cells were collected as epithelial and nonleukocyte fractions, respectively. Samples of each fraction were subjected to reverse transcription-PCR detecting β-actin, interleukin-2 receptor (IL-2r), α-fetoprotein, and hTERT mRNAs. The cases were judged to be positive, equivocal, or negative for CC cells when hTERT positivity with IL-2r negativity, hTERT positivity with IL-2r positivity, or hTERT negativity was seen in epithelial and/or nonleukocyte fractions, respectively.

Results: The dilution experiments revealed that our system could detect 10⁶–1 HeLa cells involved in 2 ml of blood. The Ber-EP4-harvested cells from cases with distant metastasis were positive for immunostaining using Hep Par 1 monoclonal antibody. CC cells were judged to be positive in 29 of 55 (53%) HCC cases. On the contrary, no cases without HCC were determined to be positive. The frequency of positivity was significantly correlated with disease extent of HCC.

Conclusions: These results strongly suggest that detection of hTERT mRNA after immunomagnetic separation is a specific and sensitive tool to detect CC cells and that it would provide useful source for further investigation of cancer metastasis.

INTRODUCTION

HCC is a major cause of death in patients with chronic liver diseases associated with hepatitis B and/or C viruses. Recently, several promising therapeutic options have become available for HCC treatment such as radiofrequency ablation (1, 2) or liver transplantation (3). Although these means are probably effective in controlling the local invasion of HCC, they are not effective in controlling distant extension of the disease. Predictive indicators are necessary to estimate the risks of metastatic recurrent diseases, especially before transplantation, in which donor depletion and cost expansion raise social problems.

Patients with various types of cancers have risks of recurrence even after curative resection (4–7) or organ transplantation (3, 8). Because only the residual cancer cells outside of the liver of a recipient can cause recurrence after liver transplantation in HCC patients, CC cells must cause the high incidence of recurrent diseases (3, 8). Therefore, the development of a strategy to detect CC cells would have a strong impact on clinic by repressing redundant therapies. Detection of AFP mRNA in peripheral blood of HCC patients by nested RT-PCR has been reported to be useful as a prognostic predictor of HCC (9–14). It was reported, however, that AFP mRNA was detected even in DC cases (10, 13–16). Furthermore, it was unclear what types of cells were resources of the detected AFP mRNA because AFP mRNA was detected in the mononuclear cell fraction.

Telomerase is a reverse transcriptase implicated in de novo synthesis of GGTAG telomeric DNA onto chromosomal ends to stabilize genomic integrity, concomitant with immortality in cancer cells (17–19). Aggressive investigations revealed that telomerase is reactivated in approximately 85% of various types of malignant tumors but that it is inactive in most nonneoplastic somatic cells (19, 20). hTERT is a catalytic component of...
reverse transcriptase (21), and hTERT expression is rate-limiting for telomerase activity (22). Therefore, we examined whether the evaluation of hTERT mRNA is useful for detection of CC cells. Because it was reported that activated lymphocytes involved in CD25-positive fraction express hTERT mRNA without malignant transformation (23, 24), we separated epithelial cells from blood cells using immunomagnetic beads bound to antiepithelial or anti-pan-leukocyte antibodies. These separation procedures made it possible to visualize the cells responsible for the detected mRNA. Here we report a buildup of a highly sensitive and specific system to detecting CC cells. This system improved inherent problems in previous reports (9–16, 25–27) on the basis of hTERT expression of immunomagnetically separated epithelial cells. Clinical usefulness was evaluated by adapting the system in HCC patients.

**MATERIALS AND METHODS**

**Patients.** Ninety-five cases were enrolled in this study: 55 cases with HCC at various stages; 20 DC cases; and 20 healthy volunteers without chronic liver disease. The characteristics of the patients are summarized in Table 1. HCC was histologically diagnosed using surgically obtained specimens in 22 cases or ultrasound-guided liver biopsy specimens in 7 cases. The other HCC cases were clinically diagnosed using several imaging modalities such as computed tomography during hepatic arteriography. The disease extent of HCC was classified according to two criteria: the modified TNM staging classification by the American Liver Tumor Study Group (Table 2; Ref. 28); and the criteria by the Milan group (29). When computed tomography and magnetic resonance imaging depicted homogenous increments of arterial blood flow in accessory nodules smaller than the main tumor, these accessory nodules were diagnosed as intrahepatic metastatic lesions. Informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by Niigata University human research committee.

**Immunomagnetic Separation.** Two ml of peripheral blood were drained and anticoagulated with EDTA before various treatments and/or liver biopsy. The samples were immediately diluted with the same volume of modified PBS (PBS containing 0.5 mM EDTA and 1% BSA) and separated into two aliquots (1 and 3 ml) for negative and positive selection of epithelial cells, respectively. For the negative selection, 8 × 10⁷ immunomagnetic beads covalently coated with anti-CD45 monoclonal antibodies (Dynabeads M450 CD45; Dynal A.S., Oslo, Norway), which react to pan-leukocytes, were added and gently rotated at 4°C for 30 min. The cells that bound to the beads were harvested using a magnetic field as a L-fraction. The cells in the supernatant were also collected by centrifugation at 3000 rpm for 5 min as a NL-fraction. For the positive selection, 2.4 × 10⁷ immunomagnetic beads covalently coated with Ber-EP4 monoclonal antibodies (CELlection Epithelial Enrich; Dynal A.S.) were mixed with 1 ml of 3 ml of the diluted blood. Ber-EP4 antibody recognizes various types of normal and malignant epithelial cells including approximately 67% of HCC (30). Incubation and collection were performed in the same manner as described in the negative selection. The cells in the supernatant were collected as a NE-fraction. The harvested cells in a magnetic field were resuspended with the 1-ml residual of the diluted sample, and the cells were harvested again. The same procedure was performed once more, and final harvested cells were collected as an E-fraction. Finally, the cells of the E- and L-fractions were washed twice with 1 ml of modified PBS to clear up RBCs and resuspended in 300 μl of modified PBS.

**Microscopic Examination.** E-fractions were subjected to microscopic examination from two cases with distant metastatic lesions and five healthy volunteers. After dilution of 60 μl of each E-fraction to 2 ml with modified PBS, an aliquot of 200 μl was cytocentrifuged onto a slide glass using a cytopsin. After fixation by 100% ethanol, May-Giemsa staining and immunostaining were performed. As for immunostaining, endogenous

### Table 1 Characteristics of patients

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>Gender (M/F)</th>
<th>Age (mean ± SD)</th>
<th>Liver cirrhosis (B/C/B&amp;C/Alcohol)⁷</th>
<th>Chronic hepatitis (B/C/B&amp;C)</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy volunteer</td>
<td>20</td>
<td>14/6</td>
<td>57 ± 12</td>
<td>0/0/0/0</td>
<td>0/0/0</td>
<td>0</td>
</tr>
<tr>
<td>DC⁶</td>
<td>20</td>
<td>9/11</td>
<td>59 ± 14</td>
<td>3/5/0/4</td>
<td>1/5/0</td>
<td>2⁶</td>
</tr>
<tr>
<td>HCC</td>
<td>55</td>
<td>39/16</td>
<td>64 ± 11</td>
<td>6/21/3/2</td>
<td>4/14/1</td>
<td>4</td>
</tr>
</tbody>
</table>

⁴ B, hepatitis B virus associated; C, hepatitis C virus associated; B&C, hepatitis B and C viruses associated.
⁵ One patient had liver cirrhosis due to unknown etiology, and another had primary biliary cirrhosis.
⁶ Two patients had no chronic liver diseases, and the other two patients had received liver transplant surgery.

### Table 2 Modified TNM staging classification⁸

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>T₀</td>
<td>Tumor not found</td>
</tr>
<tr>
<td>T₁</td>
<td>1 nodule &lt; 2.0 cm</td>
</tr>
<tr>
<td>T₂</td>
<td>1 nodule = 2.0–5.0 cm; 2 or 3 nodules, all ≤ 3.0 cm</td>
</tr>
<tr>
<td>T₃</td>
<td>1 nodule &gt; 5.0 cm; 2 or 3 nodules, at least one &gt; 3.0 cm</td>
</tr>
<tr>
<td>T₃₉</td>
<td>4 or more nodules, any size</td>
</tr>
<tr>
<td>T₆</td>
<td>T₂, T₃, or T₃₉ plus gross intrahepatic portal or hepatic vein involvement as indicated by CT, MRI, or US⁹</td>
</tr>
<tr>
<td>N₁</td>
<td>Regional (porta hepatis) nodes involved</td>
</tr>
<tr>
<td>M₁</td>
<td>Metastatic disease, including extrahepatic portal or hepatic vein involvement</td>
</tr>
</tbody>
</table>

⁸ by American Liver Tumor Study Group (Ref. 28).
⁹ CT, computed tomography; MRI, magnetic resonance imaging; US, ultrasonography.
peroxidase activity was inhibited by immersing the slides in 3% hydrogen peroxide for 45 min. The slides were incubated with murine monoclonal antibodies specific for human hepatocyte (anti-human hepatocyte, Hep Par 1; Dako, Copenhagen, Denmark) at room temperature for 60 min and successively incubated with the biotinylated goat anti-murine IgG for 30 min. Finally, the reaction was developed with diaminobenzidine solution after incubation with avidin-biotin peroxidase complex for 30 min at room temperature.

**RT-PCR.** Total RNA of each fraction was isolated using IsoGen LS (Wako, Osaka, Japan) according to the manufacturer’s instructions and resuspended in 20 μl of water treated with diethyl pyrocarbonate. After denaturation, cDNA synthesis was performed by incubating 5.6 μl of aliquots in a 20-μl reaction mixture consisting of 200 pmol of random hexaoligonucleotides and 7 units of RAV-2 reverse transcriptase (Takara, Otsu, Japan). Three μl of synthesized cDNA were subjected to PCR reaction after diluting the products to 60 μl with diethyl pyrocarbonate-treated water. For amplification of hTERT cDNA, nested PCR was executed. In the first PCR, 369-bp fragments from a 1435–1803 nucleotide position (GenBank accession number AF018167), which is not involved in reported alternatively spliced regions (31), were amplified in 10 μl of a reaction mixture containing 5 pmol of each primer (5′-AGTTGTAAGGCTTCTGTCGCCG-3′ and 5′-TGCTCAGACACTCTCCTCCG-GTAG-3′), 0.2 mm deoxynucleoside triphosphates, and 0.5 unit of Taq DNA polymerase (Takara). Amplification was performed using a Thermal cycler MP (Takara) with 35 cycles as follows: denaturation at 98°C for 15 s; annealing at 65°C for 30 s; and extension at 72°C for 30 s with a final extension at 72°C for 5 min. Three μl of 100-fold diluted first PCR products were subjected to a second PCR using primers 5′-CACGCCAACAGGAACGGCCG-3′ and 5′-CCTGAGGAGCCTCAGCGACGTA-3′, which amplify 235-bp fragments at a 1496–1730 nucleotide position. The second PCR program was the same as that of the first PCR, except for the annealing temperature at 67°C.

A 491-bp fragment of α chain of IL-2r cDNA at 422–912 nucleotide position was amplified in a 10-μl reaction mixture using primers 5′-AATGCAACAAGCTCCTGGCAACTC-3′ and 5′-GGCCACTGCTACCTGGTACCT-3′. The amplification cycles consisted of 35 cycles, denaturation at 94°C for 30 s, annealing at 65°C for 30 s, and extension at 72°C for 30 s. For hTERT-positive fractions, 3 μl of 100-fold diluted first PCR products were subjected to a second PCR performed in the same manner as the first PCR. A 626-bp fragment of β-actin cDNA specific for active β-actin gene was amplified in the same manner as the previous report (32). Nested PCR for amplification of AFP messages was performed in the same way as the previous report (10) using the cDNA prepared for hTERT evaluation. After separation through 6% polyacrylamide gels, all PCR products were visualized by ethidium bromide staining.

**Dilution Experiments of Immunomagnetic Separation.** To examine the sensitivity and specificity of our system, serial dilution experiments were performed. HeLa cells (American Type Culture Collection, Manassas, VA) were suspended in peripheral blood drained from a normal volunteer at a concentration of 10^2–10^5 in every 2 ml with every 10-fold dilution. Considering dilution errors, the dilutions for 10^3 and 10^4 were additionally examined five times. Furthermore, SR cells (American Type Culture Collection), which are lymphoma cells that constitutively express IL-2r (CD25; Ref. 33), were suspended in modified PBS at a concentration from 10^6 to 10^9 in every 2 ml with every 10-fold dilution. The cells were harvested using Ber-EP4 antibodies from each dilution, and both messages of hTERT and IL-2r were detected by RT-PCR. In addition, E-fractions from 10^3 HeLa and 10^6 SR cells were cytocentrifuged and stained by the May-Giemsa method.

**RESULTS**

**Sensitive and Specific Capture of the Diluted Cancer Cells.** The dilution experiments using HeLa cells revealed that hTERT mRNA was detected in all samples involving more than 10^3 cells (Fig. 1A) and in one of five samples including 10^0 cell (Fig. 1B) in 2 ml of peripheral blood. IL-2r mRNA was not detected in any samples, regardless of the number of added HeLa cells. On the other hand, hTERT messages were detected only in the E-fraction from 10^6 SR cell dilution concomitantly with IL-2r messages (Fig. 1C).

Microscopic observation of E-fraction from 10^6 HeLa cell dilution clearly showed cells surrounded by plural magnetic beads (Fig. 1D). The cells were characterized by their largeness in comparison with the circulating leukocytes, an enormous nucleus and a large ratio of nucleus to cytoplasm. Some cells were attached to the magnetic beads and torn into smaller fragments. There were 10 or more cells detected in every slide. Although precise quantitation was difficult, it was estimated approximately 80% of the added HeLa cells well captured. No leukocytes were, however, observed in any fields studied. On the other hand, any cells were not observed in E-fraction from 10^3 SR cell dilution.

**Capture of Circulating Hepatocytes in Patients with HCC.** Microscopic observation revealed atypically large cells with an enormous nucleus and a large ratio of nucleus to cytoplasm in E-fractions from HCC cases. On the other hand, no cellular integrant was recognized in E-fractions from all five normal volunteers. To clarify whether these captured cells were hepatocytes or not, immunohistochemistry was performed using Hep Par 1 monoclonal antibody. The results showed that all captured cells were clearly recognized by this antibody, as hepatocytes or not, immunohistochemistry was performed using Hep Par 1 monoclonal antibody. The results showed that all captured cells were clearly recognized by this antibody, as shown in Fig. 2A. Fragmented cell debris, which was bound to magnetic beads, was also observed. Although it was difficult to estimate precisely, 5–10 cells were detected in every slide, and the fragmentation frequency was higher than that in the dilution experiments. No positive reactions were observed on normal leukocytes using Hep Par 1 antibody (data not shown). No leukocytes were observed in any samples examined.

The expression of hTERT was detected in all E-fractions from these HCC cases (Fig. 2B). In case 1, hTERT messages were also detected in the NE-fraction. On the contrary, the
Fig. 1 Microscopic observation and detection of hTERT and IL-2r messages in serial dilution using HeLa and SR cells. A, the amplified products of hTERT and IL-2r cDNAs were separated through 6% polyacrylamide gels and stained with ethidium bromide. The products of hTERT were detected at an estimated size of 235 bp as indicated by a closed triangle in the harvested fractions using Ber-EP4 antibody, which involved 10^4, 10^5, and 10^6 HeLa cells in 2 ml of blood from a normal volunteer (as shown in Lanes 3–5, respectively). No products were visualized in Lanes 1 and 2, in which no and 10^6 HeLa cells were involved in 2 ml of blood, respectively. Lane M shows 100-bp ladders with the 200-bp band visible at the bottom of the gel (top panel). For amplification of IL-2r cDNA, generating 491-bp products (open triangle), no products were generated even by a nested PCR in any samples, regardless of the number of added HeLa cells. Lane M shows 100-bp ladders including a spike at 500 bp (bottom panel). B, the dilution experiments were additionally examined five times for 10^6 HeLa cell. hTERT mRNA was detected in only one of five samples, as indicated by a closed triangle (top panel). IL-2r mRNA was not detected in any samples (bottom panel). Lanes M of the top and bottom panels show the same 100-bp ladders as shown in the top and bottom panels of A, respectively. C, amplified products of hTERT (top panel) and IL-2r (bottom panel) were concomitantly detected in the sample, which was harvested from 10^6 SR cells in 2 ml of modified PBS using Ber-EP4 antibody as shown in Lane 7. On the other hand, no products were detected in 10-fold dilutions of SR cells from 10^6 to 10^4 in 2 ml of modified PBS as shown in Lanes 1–6, respectively. Lane 8 shows the products detected in the supernatant of the same sample used in Lane 7. Lanes M of the top and bottom panels show the same 100-bp ladders as seen in the top and bottom panels of A, respectively. D, the cells, which had an enormous nucleus and a large ratio of nucleus to cytoplasm, were observed in the Ber-EP4-captured fraction as shown in the left panel (May-Giemsa stain; original magnification, ×400). The size of the cell was estimated as approximately 16 × 20 μm from the size of a magnetic bead of 4 μm. Some of the cells were fragmented with attached beads, as shown in the right panel.

Fig. 2 Microscopic observation and detection of hTERT and IL-2r messages in two HCC cases with distant metastasis. A, two large cells were aggregated and surrounded by many immunomagnetic beads conjugated with Ber-EP4 antibody as shown in the left panel. The cytoplasm was clearly stained by immunohistochemical study using Hep Par 1 monoclonal antibody. The size of the cells was estimated to be approximately 24 × 20 μm from the size of a magnetic bead of 4 μm. Some of the cells were fragmented with the beads and holes. The hole was similar in size to the bead, as indicated by a closed triangle (right panel; original magnification, ×400). B, in aliquots from the same fractions used in Hep Par 1 immunostaining as shown in A, hTERT messages were detected in both cases without IL-2r messages as shown in Lanes E. On the other hand, IL-2r messages were amplified in the supernatant of E-fractions as shown in Lanes NE. The amplified products of hTERT were also detected in Lane NE of case 1, suggesting the existence of activated lymphocytes with telomerase activity or capturing failure of cancer cells. E represents the harvested fraction captured by Ber-EP4 antibody. NE represents the supernatant recovered from preparation of E.

amplified products of IL-2r mRNA were generated in NE-fractions of both cases, but not in E-fractions. In case 1, AFP expression was also detected in the E-fraction (data not shown).

Detection of CC Cells in HCC Cases at the Various Clinical Stages. Each message was detected in 55 cases with HCC at various clinical stages, 20 DC cases, and 20 healthy volunteers. The expression of β-actin was detected in all fractions in most cases. The intensity of the amplified products of β-actin messages was weak in E-fraction compared with that in the other fractions from most cases.

Because hTERT messages were detected in the IL-2r-positive sorted lymphocytes from a normal volunteer (24), we defined the existence of CC cells as in the case, in which hTERT mRNA was detected in E-fraction, but IL-2r mRNA was not detected in E-fraction. We also performed counterexperiments because Ber-EP4 monoclonal antibody does not recognize all epithelial cells. The observation that hTERT is expressed without IL-2r expression in the NL-fraction was also defined as the existence of CC cells. Consistently, CC cells were judged to be negative when hTERT mRNA was not detected in either the E- or NL-fraction. All of the other cases were judged to be equivocal in consideration of contamination of the activated lymphocytes possessing telomerase activity (23, 24). Representative positive, negative, and equivocal cases are shown in Fig. 3.
No cases without cancer (including 20 DC cases) were diagnosed to be positive (Table 3). In HCC cases, hTERT messages were detected in the epithelial fractions, Lanes E and/or NL, without the products of IL-2r messages. These cases were judged to be CC cell positive. In case 5, no amplified products of hTERT mRNA were observed in any of the epithelial fractions (Lanes E and NL). This case was judged to be CC cell negative. In case 6, although hTERT expression was detected in the epithelial fraction, Lane NL, IL-2r expression was also detected in the same fraction. This case was determined to be equivocal. The signals of β-actin in epithelial fractions were faint compared with those of the other fractions. The message of IL-2r was detected in all non-epithelial fractions, Lanes NE and L, of all examined cases. In several cases, hTERT message was detected in the non-epithelial fraction, as shown in Lane L in case 4 and Lanes NE and L in case 5. E and L represent the harvested fractions captured by Ber-EP4 and anti-CD45 antibodies, and NE and NL represent the supernatants recovered from preparation of E and L, respectively. Lane M shows 100-bp ladders including a spike at 500 bp.

There was a positive correlation between the existence of CC cell and the positivity of CC cells with statistical significance ($P = 1.9 \times 10^{-3}$; Table 4). The frequency of CC cell positivity was highly correlated with disease extent from 25% in $T_1$ to 88% in $M_1$ ($P = 0.0068$). All HCC nodules in CC cell-positive cases at $T_1$ and $T_2$ were determined to be hypervascular lesions. On the other hand, CC cells were negative in all seven cases (six cases in $T_1$ and one case in $T_2$) that had only well-differentiated HCC nodules. The frequency of CC cell positivity in the cases that did not meet the criteria by the Milan group (73%) was significantly higher than that in the cases that met the criteria (28%; $P = 0.0011$). The existence of IM and CC cell positivity were significantly associated ($P = 0.0062$).

Detection of AFP mRNA in the E- and/or NL-Fraction. AFP mRNA was not detected in any cases without HCC, whereas it was detected in 8 of 55 HCC cases (15%). The positivity was significantly associated with the existence of HCC ($P = 0.019$), but not with the disease extent as classified by the modified TNM ($P = 0.13$; Table 4). AFP mRNA was not detected in five of eight HCC cases with distant metastasis in our study. The frequency of CC cell positivity in the cases that did not meet the criteria by the Milan group (23%) was not significantly higher than that in the cases that met the criteria (4%; $P = 0.059$). The existence of IM and CC cell positivity by detection of AFP mRNA were significantly associated ($P = 0.018$).

DISCUSSION

To date, CC cells in HCC cases were evaluated mainly by detecting AFP messages. As in the cases with IM in this study, detection of AFP mRNA in peripheral blood could provide useful information in the selected cases. However, AFP messages are expressed not only in HCC but also in normal hepatocytes, and the messages were detected in the peripheral blood of DC cases (10, 15, 16). In addition, the positivity of AFP mRNA was very low in our study, although the RNA extraction procedure was different from that used in previous reports. On the other hand, the dilution experiments for hTERT detection indicated that our system had a sensitivity that could detect $10^{10}$-1 cancer cells in 2 ml of blood. There are controversial reports regarding the issue of whether hTERT mRNA is expressed in noncancerous hepatocytes (34–36). However, no signals were reported by in situ hybridization using liver tissues from chronic hepatitis to early cirrhosis (35), which usually would be subjected to evaluation of prognosis of HCC as in the cases of our study. In addition, the Ber-EP4 antibody used in positive selection does not react with normal hepatocytes. Thus, the possibility may be very low in our setting that false positive occurred. Indeed, CC cells were not detected in any of the cases without cancers, even those affected by chronic viral hepatitis. The correlation between CC cell positivity and disease extent also suggests the specificity of our system. We observed no metastatic recurrent diseases within 1 year after curative surgical resection in all four CC cell-negative cases including two liver transplant recipients, whereas all three CC cell-positive liver transplant recipients developed metastatic recurrences within 3 months after transplantation (data not shown).

The separation of epithelial cells in peripheral blood has been reported using Ber-EP4 antibody conjugated immunomagnetic beads (5, 37–39). Inevitably, there will always be a number of leukocytes coisolated by this procedure. Our dilution study demonstrated that constitutively IL-2r-positive SR cells were harvested by positive selection only when more than $10^6$ cells were contaminated in 2 ml of PBS. The concentration of $10^6$ cells in 2 ml is approximately 10 times higher than that of IL-2r-positive activated lymphocytes that would be usually detected in vivo. Taken together, our results strongly suggest that this method has sufficient specificity to detect CC cells in a clinical setting.

Ber-EP4 antibody cannot recognize all epithelial cells; for example, only 67% of HCCs are reactive, and normal hepatocytes are nonreactive (30). The separation would miss the circulating HCC cells if only Ber-EP4 antibody was used. Therefore, we performed negative selection using immunomagnetic beads bound to anti-CD45 antibody (40). This combination led
to an elevation of positivity from 44% to 53%. However, the condition of negative selection in our system should be further optimized because both hTERT and IL-2r messages were positive in 13 cases (24%) of NL-fraction, suggesting the existence of leukocytes slipping from anti-CD45 antibody. Furthermore, the intensity of the amplified products of /H9252-actin messages was weaker in E-fractions compared with that in NL-fractions. If the cells in NL-fractions consisted of only cancer cells, the intensity must be higher in E-fractions derived from 1.5 ml of blood in comparison with in NL-fractions derived from 0.5 ml of blood. This observation suggests that there were not only leukocytes but also nonneoplastic nonleukocytes and/or hTERT-negative cancer cells, which were nonreactive to Ber-EP4 antibody, in NL-fractions. The cells consisting of NL-fractions should be characterized by further examinations.

The usefulness of quantifying telomerase activity to diagnose CC cells was reported using a nonradioactive and semi-quantitative ELISA coupled with telomeric repeat amplification protocol (37, 38, 41). Because clinical materials are sometimes degraded and missampled, the sample quality must be evaluated before subjecting it to sensitive analyses. Especially when telomerase is used as an indicator of cancer diagnosis, lymphocyte contamination must be evaluated (24). It is relatively difficult, however, to evaluate the quality of the samples subjected to telomeric repeat amplification protocol assay. In our system, degradation and missampling were easily evaluated by amplifying /H9252-actin messages. Although hTERT messages were detected in E-fraction without /H9252-actin expression in 3 of 24 cases, higher sensitivity due to nested PCR for amplification of hTERT must cause this discrepancy. The lymphocyte contamination was not only reduced by immunomagnetic separation but also evaluated by amplifying IL-2r mRNA. Thus, our system detecting mRNA is thought to be more advantageous than the system detecting telomerase activity because false positive and negative diagnosis is theoretically estimated to be very low in our system.

There have been few reports morphologically indicating CC cells in clinical cases. The source of mRNA is highly speculative in previous studies, which detect only messages in the mononuclear cell fraction. In contrast, the captured cells in

### Table 3: Detectability of CC cells

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of cases</th>
<th>hTERT mRNA</th>
<th>AFP mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>n = 20</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>DC</td>
<td>n = 20</td>
<td>29 (53%)</td>
<td>8 (15%)</td>
</tr>
<tr>
<td>HCC</td>
<td>n = 55</td>
<td>P = 1.9 × 10^{-9}</td>
<td>0 (0%)</td>
</tr>
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</table>

### Table 4: Diagnosis of CC cells in HCC at various disease extents

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of cases</th>
<th>hTERT mRNA</th>
<th>AFP mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCC (−)</td>
<td>40</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>HCC (+)</td>
<td>55</td>
<td>29 (53%)</td>
<td>8 (15%)</td>
</tr>
<tr>
<td>Modified TNM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1, T2, and T4a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>8</td>
<td>2 (25%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>T2</td>
<td>17</td>
<td>5 (29%)</td>
<td>1 (6%)</td>
</tr>
<tr>
<td>T3</td>
<td>15</td>
<td>9 (60%)</td>
<td>2 (13%)</td>
</tr>
<tr>
<td>T4</td>
<td>7</td>
<td>6 (86%)</td>
<td>2 (29%)</td>
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<tr>
<td>M1</td>
<td>8</td>
<td>7 (88%)</td>
<td>3 (38%)</td>
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<td>Criteria by Milan Group</td>
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<tr>
<td>Meets criteria</td>
<td>25</td>
<td>7 (28%)</td>
<td>1 (4%)</td>
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<tr>
<td>Does not meet criteria</td>
<td>30</td>
<td>22 (73%)</td>
<td>7 (23%)</td>
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<td>Intrahepatic metastasis</td>
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<tr>
<td>Negative</td>
<td>21</td>
<td>6 (29%)</td>
<td>0 (8%)</td>
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<tr>
<td>Positive</td>
<td>34</td>
<td>23 (68%)</td>
<td>8 (24%)</td>
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</tbody>
</table>

*Modified TNM staging classification by American Liver Tumor Study Group (Ref. 28).
the E-fraction of our system were highly likely to be HCC cells based on the following three reasons: (a) the cells were separated using immunomagnetic beads conjugated by Bet-EP4 antibody, which does not react to normal hepatocytes; (b) the fraction involved hTERT-positive, IL-2r-negative cells; and (c) the cells were morphologically consistent as cancer cells and were positive in immunohistochemical study using Hep Par 1 monoclonal antibody, which is highly specific for hepatocytes and HCC cells (42).

Recently, it has often been reported that the traditional International Union Against Cancer TNM classification (43) does not have sufficient prognostic power to discriminate patients with respect to risk for HCC recurrence after liver transplantation (44, 45). Therefore, we categorized the disease extent of HCC according to the modified TNM (28) and the criteria of the Milan group (29). In our study, CC cell positivity was significantly associated with the extent of HCC. In particular, CC cell positivity in patients with intrahepatic vascular involvement (T4a; 86%) was nearly equal to that in patients with extrahepatic metastasis (M1; 88%). We consider that the 86% (in T4a) and 88% (in M1) positive diagnoses mean the existence of CC cells in almost all cases in these stages because telomerase activity and hTERT expression are only seen in approximately 85% of HCC (19, 20).

IM has been considered to occur via the intrahepatic portal vein (7, 46). Intrahepatic recurrence of HCC after liver transplantation, however, must develop via the systemic circulation (4). The present data showed that CC cell positivity in patients with IM was significantly higher than that in patients without IM, which suggests IM via the systemic circulation. Surprisingly, however, CC cells were detected not only in the cases at advanced stages but also in 2 of 8 T1 cases and in 5 of 17 T2 cases. All seven CC cell-positive T1/T2 patients have not developed any extra- or intrahepatic metastases within 1 year after treatment. Detection of AFP mRNA was also reported in the cases at the early stages (10, 11). Taken together, it is suggested that there may be two types of CC cells: cells detached from the liver with and without capability of implantation. Further characterization of CC cells is necessary in this type of examination to provide more accurate information in the clinic. Our system will serve as a very useful source for investigation to clarify metastatic mechanisms such as cell adhesion and local immunity at the attached sites by providing HCC cells detached from the liver.

In summary, we developed a sensitive and specific system to detect CC cells and evaluated its usefulness in HCC cases. Because only universal molecules Bet-EP4 and hTERT were used for separation and detection of cancer cells in our system, it is strongly suggested that the system could be applied not only to HCC but also to various types of cancers under the same conditions. Although further investigation is necessary, the present system for the detection of CC cells may improve prognostic estimates and appraisal of therapeutic outcomes.

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