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Detection and Quantitation of Circulating Cancer Cells in the Peripheral Blood of Lung Cancer Patients[1]

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ABSTRACT

Detection and quantitation of circulating cancer cells in peripheral blood may improve cancer staging and monitoring. This study explored the feasibility of using circulating cancer cell detection in peripheral blood for the rapid assessment of chemotherapeutic response. Cytokeratin 19 mRNA was amplified by nested reverse transcriptase-PCR in the peripheral blood of 29 healthy volunteers, 33 pneumonia patients, and 86 lung cancer patients. Circulating cancer cells in the peripheral blood were semiquantitatively determined by taking the ratio of cytokeratin 19 band intensity from the second round of nested PCR to the glyceraldehyde-3-phosphate dehydrogenase band intensity from the first round of PCR amplification. The detection limit of the method was 1 cancer cell in 10^7 peripheral blood mononuclear cells. The positive detection rate was 40% for lung adenocarcinoma patients of all stages, 41% for squamous carcinoma patients of all stages, and 27% for small cell lung cancer patients. Only one control sample from a pneumonia patient showed a positive result (1.6%). The quantitative method reliably and sensitively estimated cancer cell numbers in the peripheral blood of lung cancer patients. Serial measurement of the relative number of circulating cancer cells correlated with the tumor burden and treatment response of patients. This method may help rapidly assess the efficacy of anticancer treatment, redefine cancer staging, and facilitate the design of better therapeutic strategies for the treatment of cancer patients.

INTRODUCTION

Many previously unmanageable cancers can now be cured by modern treatment methods, provided that diagnosis is made early. All current methods for cancer diagnosis and metastasis detection rely on imaging principles, including radiography, computed tomography, magnetic resonance imaging, bone scintigraphy, and sonography. These techniques require human interpretation and may be influenced by human error. Furthermore, the tumor size detection limit of these techniques is ~1 cm, which represents 10^9 cells or a mass of 1 g (1). A more sensitive detection method would help early diagnosis and could improve the survival rate and prognosis of cancer patients.

Several research groups have recognized the potential of detecting circulating cancer cells to diagnose cancer and metastasis. Immunocytology (2), flow cytometry (3), and PCR (4–6), have been used to detect cancer cells in peripheral blood. These techniques use either antibodies or specific probes to oncogenes or gene products and apply only to one particular type of cancer. Many cancer cells, however, metastasize before the primary tumor is diagnosed, and information regarding which oncogenes or suppressor genes are expressed is usually unavailable. Therefore, it is necessary to develop a universal method to detect all kinds of circulating cancer cells in peripheral blood.

Cytokeratin (7) is a characteristic intermediate filament of epithelial cells and their malignant counterparts and has been suggested as a marker for the sensitive detection of circulating cancer cells by RT-PCR[3] (8–10). The mRNA of CK19 has been successfully used to detect circulating cancer cells in breast cancer patients with a sensitivity of 10 cancer cells in 10^6 PBMN cells (11). However, it was also cautioned that low levels of CK19 were expressed in PBMN cells, which could reduce the specificity and hamper the use of CK19 as a detection marker (12).

Here, we identify possible pitfalls of using cytokeratin as a detection marker and refine the method to be more specific and sensitive. Furthermore, we describe a semiquantitative assay for estimating the number of circulating cancer cells. This method may provide a useful tool to rapidly assess the efficacy of chemotherapy and optimize the outcome of cancer treatment.

MATERIALS AND METHODS

Cell Lines and Patients. Cancer cell lines PC9 (lung adenocarcinoma; Ref. 13), CRL-5802 (lung squamous carcinoma, obtained from American Type Culture Collection), CRL-5809 (lung small cell carcinoma, obtained from American Type Culture Collection), CL1-0, and CL1-5 (lung adenocarcinoma; Ref. 14) were used in model studies to define the detection sensitivity of the assay.

The study population consisted of 86 patients in the National Taiwan University Hospital with histologically documented lung cancer. Staging procedures included chest radiography, bronchoscopy, thoracic computed tomography, sonography, bone scintigraphy, and bone marrow aspiration. The mean follow-up period of the lung cancer patients was 113 ± 76 days. The control population consisted of healthy college students and graduates, ages 20–29 years; persons enrolled in the National Taiwan University Hospital health examination program; and volunteers with no history or present diagnosis of malignancy or other diseases. To determine whether patients suffering from infectious diseases with elevated leukocyte counts and necrotic cells from inflammation would produce false-positive results, the study also included 33 patients with lung infections but with no diagnosis of malignancy. The characteristics of the lung cancer patients, pneumonia patients, and healthy controls are summarized in Table 1.

Sample Collection and RNA Preparation. After informed consent was obtained, peripheral blood samples were withdrawn from the veins of the patients and healthy individuals with Vacutainer (Becton Dickinson, Rutherford, NJ) blood-collecting needles and tubes containing EDTA. Two samples were collected from each subject, with 1–2 ml of peripheral blood in the first tube and 3–5 ml in the second tube. The first tube, which could be contaminated with epithelial cells picked up by the needle when it pierced through the skin, was then discarded, and only the second tube was assayed.

The cell lines used for model studies were spiked in peripheral blood samples collected from healthy individuals by the above method. The number of PBMN cells in each blood sample was measured with a Coulter counter (model T-540; Coulter Electronics, Hialeah, FL) to determine the volume of blood that contained 10^7 cells. The number of cancer cells spiked in the peripheral blood were counted with a Coulter counter and serially diluted for cell numbers exceeding 100. For cell numbers below 100, cancer cells were counted and picked up under a microscope using a microinjection apparatus (Narishige, Tokyo, Japan).

Total cellular RNA was extracted from the patients' or the cancer cell

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[3] The abbreviations used are: RT-PCR, reverse transcriptase-PCR; CK19, cytokeratin 19; PBMN, peripheral blood mononuclear; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CCU, cancer cell unit(s).

Table 1 *Characteristics of lung cancer patients and healthy controls*
Numbers of CK19-positive patients are shown in parentheses.

Characteristic	Lung cancer patients	Healthy controls	Pneumonia patients
No. tested	86 (32)	29 (0)	33 (1)
Sex (male/female)	57/29	19/10	24/9
Age, yr [median (range)]	66 (26–82)	56 (24–78)	73 (22–89)
Follow-up period, days	113 ± 76		
Histopathology			
Adenocarcinoma	47 (19)		
Squamous cell	17 (7)		
Poorly differentiated	7 (2)		
Small cell carcinoma	15 (4)		
Cancer stage			
Non-small cell carcinoma			
Stage I	6 (3)		
Stage II	4 (2)		
Stage III	34 (12)		
Stage IV	27 (11)		
Small cell carcinoma			
Limited disease	5 (1)		
Extensive disease	10 (3)		

spiked peripheral blood samples with the RNeasy Blood Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Nested RT-PCR Amplification and Detection. First-strand cDNA was generated by incubation of 1–2 μg of total RNA with 5 μM random hexamer, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM dNTP, and 200 units of reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD) in a 50-μl reaction mix for 1.5 h at 37°C.

Nested PCR was used to amplify CK19 cDNA for detection. The first round (outer) of the nested PCR was performed using 4 μl of the cDNA from the reverse transcription. The PCR mix contained 0.16 μM each CK19 outer primer (primer A, 5′-AAGCTAACCATGCAGAACCTCAACGACCGC; and primer B, 5′-TTATTGGCAGGTCAGGAGAAGAGCC; Ref. 11) and 0.24 μM each GAPDH primer (5′-TTTGGTCGTATTGGGCGCCTGGTCA and 5′-TTGTGCTCTTGCTGGGGCTGGTGGT), together with 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.1% Triton X-100, 0.28 mM dNTP, and 0.8 units of DynaZyme polymerase (Finnzymes Oy, Espoo, Finland) in a total volume of 25 μl. The PCR conditions were 5 cycles of 94°C for 1 min, 65°C for 50 s, and 72°C for 2 min and 40 s, followed by 25 cycles of 94°C for 50 s, 67°C for 50 s, and 72°C for 2 min and 40 s and a final extension at 72°C for 10 min.

For the second round (inner) of the nested PCR amplification, the reaction mix contained 1 μl of the first-round PCR product, 0.48 μM each CK19 inner primer (primer C, 5′-TCCCGCGACTACAGCCACTACTACACGACC; and primer D, 5′-CGCGACTTGATGTCCATGAGCCGCTGGTAC), 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.1% Triton X-100, 0.2 mM dNTP, and 0.64 units of DynaZyme polymerase in a total volume of 25 μl. The second PCR was cycled at 94°C for 50 s and at 72°C for 2 min for 30 rounds, followed by a final extension at 72°C for 10 min. After the PCR was completed, 5 μl of PCR product were analyzed by electrophoresis on a 1.2% agarose gel. The resulting bands were quantified and analyzed on a Macintosh computer using the public domain NIH Image program (developed at NIH and available at http://rsb.info.nih.gov/nih-image/).

RESULTS

Improved Sensitivity of RT-PCR for CK19 Detection. We tested several lung cancer cell lines for the expression of CK19, as well as the expression of other cytokeratins. CK19 was one of the highly expressed cytokeratins in all of the carcinoma cell lines tested. However, unlike other cytokeratins, such as cytokeratin 8 or cytokeratin 18, which were highly expressed in carcinoma cell lines, CK19 expression was undetectable in leukocytes after one round of PCR amplification. To obtain the best detection sensitivity while minimizing background CK19 expression from PBMN cells, we used nested PCR using the same PCR primers described previously (11), but with modified PCR conditions. The optimal annealing temperatures were found to be 5–7°C lower than the 72°C previously reported by other laboratories. At these temperatures, we achieved higher detection sensitivity without any background. As illustrated in Fig. 1, *right*, no false-positive results were obtained by lowering the PCR annealing temperature in the first-round PCR to 65°C and 67°C, respectively. The lower temperature limit was determined as illustrated in Fig. 1, *left*. CK19 bands were faintly visible in the lanes of several normal controls when the PCR annealing temperature was set at 60°C and 65°C, which were 12°C to 7°C lower than those reported by other laboratories.

Effect of Blood Sampling Method on CK19 Detection. One precaution that needs special attention is the prevention of contamination by epidermal cells during sample collection. It is possible that epidermal cells could be picked up by the needle and flushed into the syringe by the blood. Because of the variation in the skills of the person collecting the samples and the condition of the subject's blood vessels, this variable can be hard to control. To avoid the problem, we used Vacutainer collection tubes to collect two blood samples from each study subject. The contaminating epidermal cells picked up during skin piercing should be retained in the first tube and not in the second tube. This hypothesis was verified from the fact that we detected CK19-positive cells in the first but not the second sample tube of three of eight healthy controls (Fig. 2).

Sensitivity and Specificity of CK19 Detection in Lung Cancer Patients. By using the modified nested PCR conditions and the sample collection method described above, we tested the peripheral blood of 86 lung cancer patients with various types and stages of cancer, 29 healthy controls, and 33 lung infection patients (Table 1). Fig. 3 shows typical results to illustrate that, under the conditions

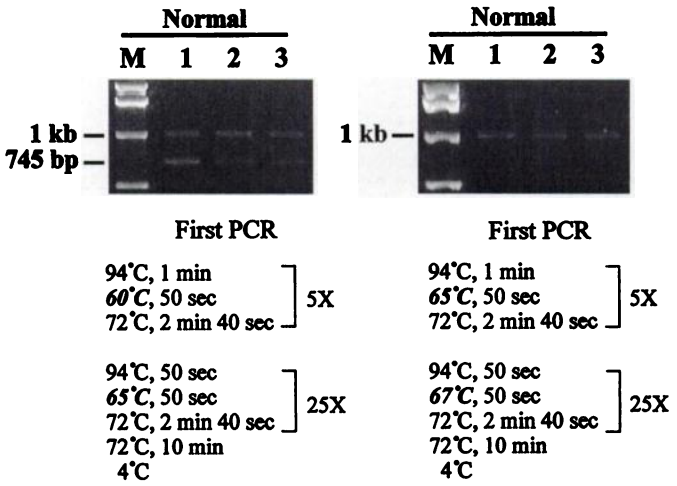

Fig. 1. Optimizing the PCR conditions by tuning the annealing temperatures. A false-positive CK19 band appeared from some of the healthy individuals after the annealing temperatures were lowered during the first PCR amplification. The cycling conditions for the second round of PCR amplification are described in "Materials and Methods."

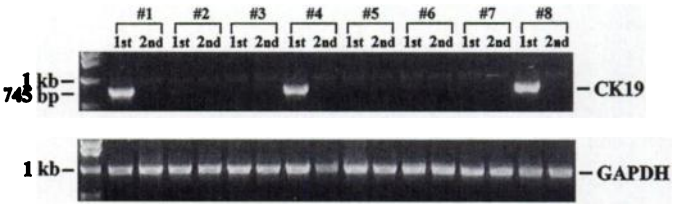

Fig. 2. Illustration of contaminating epidermal cells picked up during blood collection. *Top*, CK19 bands after two rounds of PCR amplification; *bottom*, GAPDH bands after the first round of PCR amplification. Three of the 8 controls had false-positive results in the first collection tube, but CK19-positive cells were not present in any of the second collection tubes. The faint band above the CK19 band is due to residual GAPDH DNA from the diluted first-round PCR products.

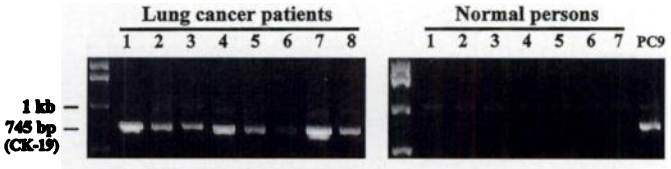

Fig. 3. Illustration of the results of the detection method. Positive CK19 amplification was obtained from various cancer patients, whereas negative results were obtained from healthy volunteers and lung infection patients. One-kb ladder markers were used as the molecular weight markers. The band above the 745-bp CK19 band is the GAPDH band. PC9 cells were added to a blood sample of a healthy individual for use as a positive control.

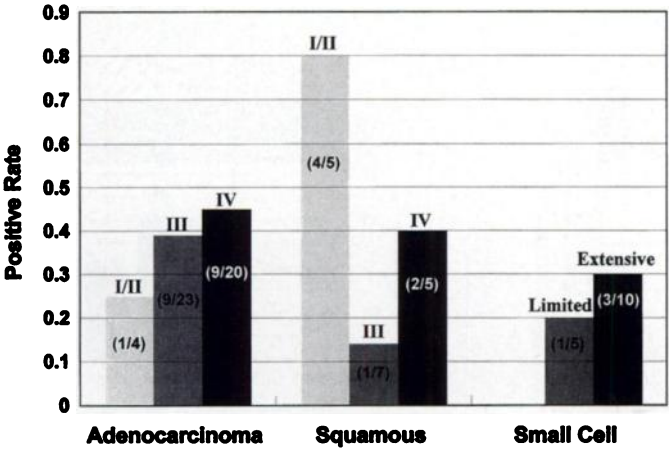

Fig. 4. A summary of the detection results for patients with various stages and types of lung cancer. Ratios of positive:total patients are indicated on the *columns*.

described, we were able to detect CK19-positive cells in the peripheral blood of lung cancer patients without the high false-positives associated with CK19 detection described in previous reports. Of 33 pneumonia patients, only 1 patient with adult respiratory distress syndrome was CK19 positive. No false-positive results were obtained with 29 healthy controls, whereas circulating cancer cells were detected in many of the lung cancer patients.

The results of circulating cancer cell detection in the lung cancer patients are summarized in Table 1 and Fig. 4. For non-small cell lung cancer patients, CK19-positive cells were detected in the peripheral blood of 5 of 10 patients with stage I and II cancer, 12 of 34 patients with stage III cancer, and 11 of 27 patients with stage IV cancer. Among these patients, the detection rate increased with cancer stage for the patients with adenocarcinoma. Twenty-five % of stage I/II, 39% of stage III, and 45% of stage IV patients had CK19-positive cells in their peripheral blood. For squamous cell carcinoma patients, four of five patients with stage I/II cancer had CK19-positive cells in their peripheral blood, which was higher than the rates in those with stage III (one of seven) and stage IV (two of five) disease. For small cell lung cancer patients, 3 of 10 patients with extensive disease and 1 of 5 patients with limited disease were also positive for circulating cancer cells.

For stage I and II patients who received surgical operation, three of six stage I lung cancer patients had CK19-positive cells in their peripheral blood before tumor resection. Two were squamous cell carcinomas and one was adenocarcinoma. None of the patients had detectable CK19-positive cells 1 week after operation. Two of four stage II patients had CK19-positive cells in their peripheral blood. Both of these patients had squamous cell carcinoma. One patient had CK19-positive cells 4 weeks after tumor resection, although the number of cancer cells decreased. No evidence of metastasis was noted during this follow-up period.

To determine the detection sensitivity of the method, PC9 lung adenocarcinoma cells were added to the blood samples of healthy controls. As shown in Fig. 5, a CK19 band could not be detected after one round of PCR amplification, whereas the GAPDH band was visible and exhibited similar intensity regardless of the number of added cancer cells. This finding is consistent with the large excess of PBMN cells (10^7) compared to cancer cells in the blood sample and indicates that the GAPDH bands were from the PBMN cells. After the second round of PCR, the integrated densities of the CK19 bands increased with increasing numbers of doped cancer cells in the blood sample. CK19, however, remained undetectable in peripheral blood without added cancer cells. At a signal:noise ratio of 3, the detection limit of the method is calculated to be 1 PC9 cancer cell per 10^7 PBMN cells.

Quantitation of Circulating Cancer Cells in Lung Cancer Patients. The detection of circulating cancer cells has many applications. Detection of circulating cancer cells in peripheral blood can provide useful information for cancer staging and for designing therapeutic strategies. Monitoring the quantity of circulating cancer cells before, during, and after the application of a therapeutic regimen was, therefore, explored.

Because the results of the RT-PCR method are obtained as the intensity of bands on agarose gels instead of absolute cell numbers, a method was developed to semiquantify cancer cell numbers from the intensities of the electrophoresis bands. The data shown in Fig. 6 were derived from the results of repetitive experiments similar to those shown in Fig. 5. GAPDH was used as an internal control because its

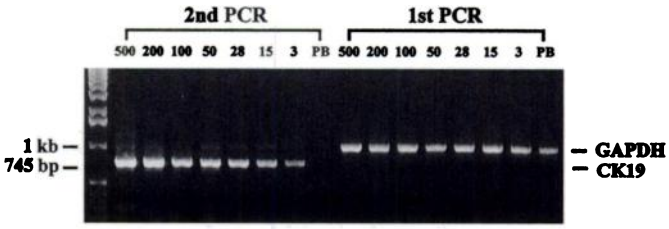

Fig. 5. Model study to determine the sensitivity of the detection method using PC9 adenocarcinoma cells. GAPDH primers were not included during the second round of PCR. *Lane numbers*, numbers of cancer cells added to 10^7 PBMN cells or ~1.5 ml of peripheral blood, on average.

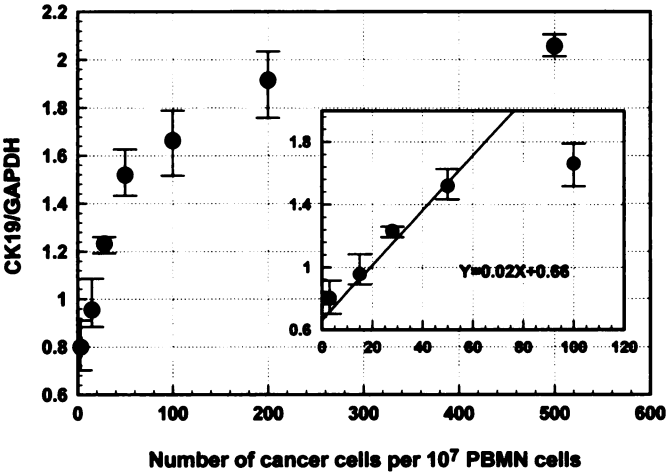

Fig. 6. Calibration of the detection method. The calibration curve was established by plotting the ratio of the CK19 band (integrated density) from the second PCR to the GAPDH band from the first PCR *versus* the number of cancer cells per 10^7 PBMN cells. *Inset*, blow-up of the first five data points. A regression line was derived from the first four data points for semiquantitative estimation of the number of circulating cancer cells in the peripheral blood of patients.

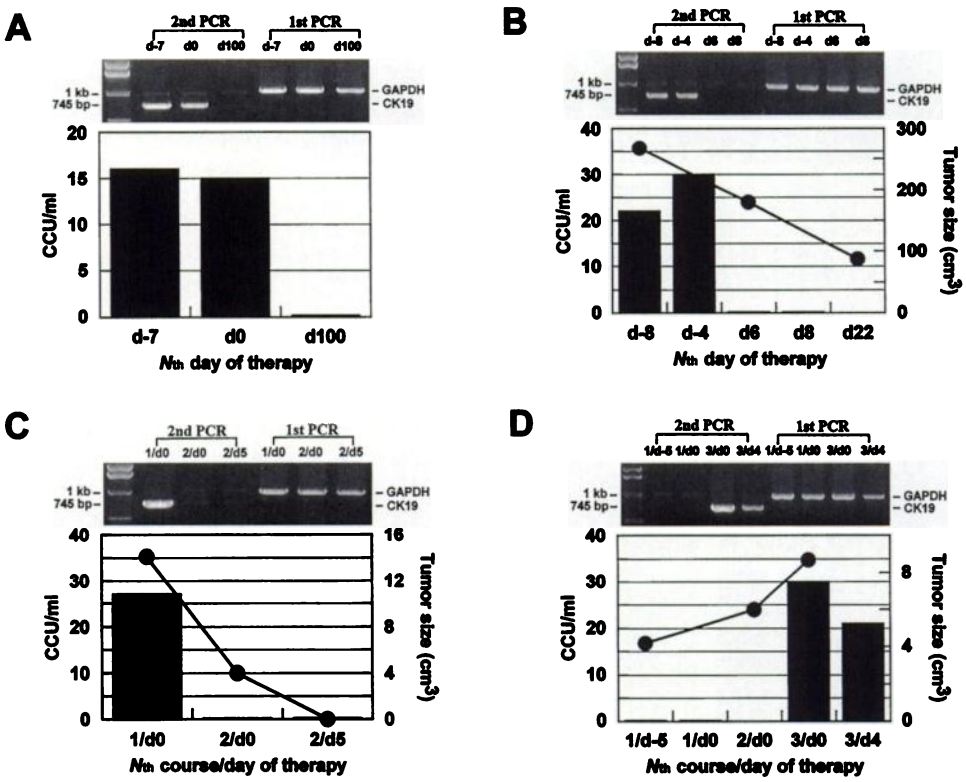

Fig. 7. Examples of the assessment of anticancer therapy efficacy by determination of the relative number of circulating cancer cells. *A*, stage I squamous carcinoma patient who received surgical resection. *B*, stage IIIb adenocarcinoma patient who received radiotherapy. *C*, small cell cancer patient with extensive disease who received chemotherapy. *D*, stage IV adenocarcinoma patient who received chemotherapy. *Columns*, estimated numbers of circulating cancer cells per ml of peripheral blood; ●, tumor sizes, determined by chest X-ray or computed tomography.

band intensity correlated with the number of PBMN cells and was not affected by the number of circulating cancer cells in peripheral blood. The dynamic range of detection was limited, as expected, by the extensive number of PCR cycles used in the nested PCR procedure (Fig. 6). The integrated densities of the CK19 bands after two rounds of PCR were divided by the integrated densities of the GAPDH bands after the first round of PCR amplification, and the results were plotted against the number of cancer cells per 10^7 PBMN cells. The ratio of the CK19 and GAPDH band intensities were then used to estimate the number of circulating cancer cells in the peripheral blood from the regression line of the first four data points in Fig. 6. To account for variations in the patients' leukocyte counts or GAPDH levels, the white cell count per ml of blood was measured before RNA was extracted. The measurement results were then calculated in terms of CCU per ml of blood. The SD of the measurements was estimated to be ~7%.

On the basis of the above semiquantitative assay approach, we explored the feasibility of applying the circulating cancer cell detection method to assess the efficacy of cancer therapy. To determine whether the number of circulating cancer cells in a patient's peripheral blood correlated with tumor progression or remission, lung cancer patients with different stages and cell types and under different therapeutic regimens were studied.

Fig. 7*A* shows the CK19 electrophoresis bands as well as the estimated number of circulating cancer cells in CCU/ml for a stage I squamous carcinoma patient who underwent surgical resection. Blood samples were collected 7 days before the operation, on the day of the operation, and 100 days after the surgical removal of the tumor. No circulating cancer cells were detected 100 days after surgery. Fig. 7*B* shows the results for a stage IIIb lung adenocarcinoma patient treated with radiotherapy. Blood samples were collected 8 and 4 days before, as well as 6 and 8 days after, radiotherapy. The size of the primary tumor was also monitored by chest X-ray 8 days before, as well as 6 and 22 days, after radiotherapy. The number of circulating cancer cells in the peripheral blood increased before therapy but dropped to zero after therapy, indicating good response to radiotherapy. The size of the tumor also decreased after therapy, verifying the efficacy of the treatment. Fig. 7*C* illustrates an example of effective chemotherapy for a small cell lung cancer patient with extensive disease. Both the number of circulating cancer cells and the size of the tumor dropped to zero after the second course of therapy. Fig. 7*D*, in contrast, is an example of the ineffective therapy of a stage IV lung adenocarcinoma patient. The size of the tumor and the number of circulating cancer cells increased from negligible to 30 CCU/ml before the third course of therapy was started. The patient received a different regimen for the third course of therapy based on the findings.

DISCUSSION

Here, we have demonstrated that circulating cancer cells can be detected by RT-PCR of CK19. We modified the method to improve the sensitivity of detection to 1 cancer cell in 10^7 PBMN. This method was also very specific, with only 1 false-positive result (1.6%) in 62 control samples. Furthermore, we also developed a semiquantitative method to estimate the relative number of circulating cancer cells in lung cancer patients. The quantitation of circulating cancer cells correlated well with the tumor cell burden of the patients. Early assessment of the therapeutic response may, therefore, be possible by serial quantitation of circulating cancer cells.

It has been reported that low-level illegitimate transcription of tissue-specific genes in nonspecific cells may limit the use of RT-PCR for detecting circulating cancer cells (12). Methods to eliminate nonspecific cells by immunobead capture of cancer cells (15) or gradient centrifugation using Ficoll-Hypaque solution have been reported and tested. In our experience, these manipulative procedures may result in loss of cancer cells and low detection sensitivity. From our experimental results, we believe that epidermal cell contamination during blood collection contributes more to the false-positive results than

does the illegitimate expression of CK19 in PBMN cells. The background CK19 amplification was undetectable on electrophoresis gel at the PCR annealing temperature of 67°C, a lower temperature than previously reported by other laboratories (11, 12), yet only one false-positive result could be detected.

The false-positive result came from a 20-year-old female patient with cerebral palsy and pneumonia, who developed severe adult respiratory distress syndrome during blood sampling. The follow-up blood sample taken after pneumonia subsided failed to demonstrate CK19 signals by nested RT-PCR. The patient was followed for 3 months, and no evidence of malignancy was noted. Whether severe tissue damage can result in leakage of viable epithelial cells into peripheral blood remains to be determined.

Here, we demonstrate an approach that is both highly sensitive and convenient to apply. The loss of cancer cells is minimized by lysing the circulating cancer cells together with the PBMN cells. The limit of detection of cancer cells in peripheral blood depends on the RNA extraction efficiency and the expression level of CK19 in the cells. By using siliconized tubes for RNA manipulation, optimized PCR conditions, and careful manipulation in each step, we were able to reach the detection limit of one PC9 cancer cell in 10^7 PBMN cells. However, among the cell lines we tested, PC9 cells had the highest CK19 expression. PC9, an adenocarcinoma cell line, had two times more CK19 than did the squamous carcinoma cell line, CRL-5802. The small cell carcinoma cell line, CRL-5809, had the lowest CK19 expression among the three cell types. These findings may also account for the different positive detection rates for patients with different types of lung cancer.

The limited number of stage I and II patients prevents us from making any conclusions but suggests that the current staging system may be inadequate to detect micrometastases. Stage I/II patients with CK19-positive cells in their peripheral blood may represent a group of people who actually have latter stage disease. Whether these patients will have worse prognosis or early recurrence is not known. We are currently recruiting more patients with earlier stages of lung cancer to analyze the clinical significance of CK19-positive cells in peripheral blood. Do these patients have micrometastases that are beyond the current detection methods? Could these patients benefit from neoadjuvant chemotherapy in addition to tumor resection? These questions require the study of a larger series of patients and longer follow-up.

Our study showed that quantitation of circulating cancer cells is possible. Because cancer cells express different levels of CK19, patients with higher CCU (PC9 equivalent) do not necessarily have more circulating cancer cells in their peripheral blood than do patients with lower CCU. Nevertheless, the semiquantitative approach is useful for measuring the relative number of circulating cancer cells in a patient's peripheral blood to monitor the effectiveness of treatment. In a limited number of patients, we demonstrated that the relative number of circulating cancer cells correlated well with the tumor burden and therapeutic response. This method highlights an alternative approach to rapidly assess the treatment response of patients. Traditionally, two courses of chemotherapy are administered before chest X-ray and computed tomography are performed to evaluate the treatment response. In contrast, the treatment response can be immediately assessed by serial quantitation of circulating cancer cells after chemotherapy. This method may, therefore, help to design more comprehensive and reasonable therapeutic regimens at earlier dates for patients.

In conclusion, nested RT-PCR for CK19 mRNA is a sensitive and specific method for detecting circulating cancer cells in lung cancer patients. Estimation of the number of circulating cancer cells is also possible by the semiquantitative method we described. This method may be helpful for early detection of micrometastasis and rapid assessment of the treatment efficacy of lung cancer patients.

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