Lung Cancer Metastatic Cells Detected in Blood by Reverse Transcriptase-Polymerase Chain Reaction and Dot-Blot Analysis

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Purpose: We analyzed the blood of patients with lung cancer at different stages of presentation for the presence of carcinoembryonic antigen (CEA) mRNA detected by reverse transcriptase-polymerase chain reaction (RT-PCR) combined with the dot-blot procedure as an indicator of micrometastatic malignant cells.

Patients and Methods: We studied 24 lung cancer patients (10 with distant metastases and 14 with no evidence of distant metastases), eight age- and sex-matched patients affected by nonneoplastic respiratory diseases (four smokers), and eight healthy subjects. We used immunohistochemistry and RT-PCR dot-blot analysis to evaluate CEA expression in the neoplastic tissue, and the RT-PCR dot-blot procedure to analyze CEA mRNA in circulating cells.

Results: The RT-PCR dot-blot procedure was highly sensitive and specific: it detected CEA mRNA in samples of RNA from lung cancer diluted 10^6-fold with RNA extracted from normal blood cells, and sequence analysis confirmed that the amplified product was CEA. CEA mRNA was found in circulating cells from eight of 10 lung cancer patients with distant metastases (diagnostic sensitivity, 80%) and in four of 14 patients with no evidence of distant metastases. Two of the latter had distant metastases within 6 months of analysis. Thus, the diagnostic specificity of the analysis toward lung cancer without distant metastases was 86%. The analysis was negative in the eight nonneoplastic patients and in the eight healthy controls.

Conclusion: The RT-PCR dot-blot analysis of CEA mRNA in blood cells seems to be a promising tool for the early detection of micrometastatic circulating cells in patients with lung cancer.

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LUNG CANCER IS ONE of the most frequent neoplasias in humans and the prognosis is closely related to the metastatic spreading of tumor cells. Approximately 40% of lung cancer patients already have distant metastases on hospital admission.[1,2] Distant metastases are also a key factor in planning the treatment of lung cancer patients, eg, non–small-cell lung cancer (NSCLC) patients are not eligible for surgery[3] and a combination of radiotherapy and chemotherapy is the standard treatment for small-cell lung cancer (SCLC) with metastatic spreading.[2] A high percentage of lung cancer patients who undergo surgery have an extrathoracic relapse of the neoplasia or are found to bear distant metastases at autopsy. Therefore, a large number of patients are understaged at diagnosis.[2] The staging procedures, currently based on abdominal ultrasonography, abdominal and brain computed tomography scan, and bone scintigraphy,[3] are highly sensitive, but not specific; they are also costly.[4] Thus, also because of the different pattern of metastases in the various histologic types of lung cancer,[3] there is no standard procedure for the staging of lung cancer patients.

Metastases develop from cancer cells circulating through the bloodstream. Therefore, a promising strategy with which to identify cancer patients that have a higher risk of metastatic relapse is to identify neoplastic cells in blood.[5-11] Neoplastic cells can be detected in blood by analyzing the expression of specific mRNA species using reverse transcription (RT) followed by amplification with the polymerase chain reaction (PCR). This procedure has been used to detect prostate-specific antigen (PSA) mRNA in patients with prostatic cancer.[5-11] It has also been used to detect albumin mRNA,[12] which is specifically expressed by liver cells, in an attempt to identify the spread of metastatic cell in patients with hepatocarcinoma.

Carcinoembryonic antigen (CEA) mRNA expression is a marker of metastatic cells in the bone marrow of patients with colorectal carcinoma.[13] The RT-PCR analysis of CEA mRNA has a higher diagnostic sensitivity than immunocytology in the early detection of metastases in these patients. A high percentage of lung cancers express CEA[14]; thus, the detection in blood of cells that express CEA, particularly in patients with lung cancer,

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could be used to recognize the metastatic process while it is still at an early stage.

We used a highly sensitive RT-PCR method, devised in our laboratory,[15] followed by dot-blot analysis to evaluate CEA mRNA expression in a population of lung cancer patients at different stages of the disease monitored for 1 year and in a control group of healthy subjects. The aim of this study was to define the potential of this approach in the early diagnosis of the metastatic process in lung cancer.

PATIENTS AND METHODS

Patients

Patients gave their informed consent to the study. The study was approved by the ethics committee of our institution. We studied 24 patients affected by lung cancer (21 men and three women); the mean age was 62.3 years (range, 45 to 81); 95% were smokers. The reference diagnosis was obtained by histologic examination of biopsies collected by fibrobronchoscopy. The histologic classification was performed according to World Health Organization (WHO) criteria[16] and patients were staged according to tumor-node-metastasis (TNM) score[17] using total-body computed tomography and bone scintingraphy with technetium 99m.

We studied 14 cases of bronchogenous carcinoma (nine squamous cell and five adenocarcinoma) with no clinical and/or instrumental evidence of distant metastases. These patients were monitored for 1 year during which computed tomography and bone scintigraphy were performed every 3 months. We also studied 10 patients affected by bronchogenous carcinoma (three squamous cell, four adenocarcinoma, and three oat cell cancers) with clinical and instrumental evidence of distant metastases. In addition, we studied a control group of sex- and age-matched patients affected by nonrespiratory diseases (four of whom were smokers) and a group of healthy volunteers ($n = 8$) from our research groups (< 40 years, with no clinical sign of pulmonary disease). For each subject, we collected two blood samples by venipuncture using the Vacutainer system (Becton Dickinson, Maylan Cedex, France), one in edathamil (EDTA) tubes, which were immediately processed for RNA extraction and for the analysis of CEA mRNA, and the other in tubes with no anticoagulant for the analysis of serum CEA. In addition, for each lung cancer patient we collected two biopsy samples, one for histology and immunohistochemical analysis of CEA; the other, collected in tubes that contained guanidinium thiocyanate to prevent degradation by the RNAses,[18] was processed within 2 hours of collection for RNA extraction and CEA mRNA analysis. As a control of the RNA extraction, all the biopsy and blood samples were analyzed for superoxide dismutase (SOD) housekeeping mRNA. The study was performed double-blind (laboratory *v* pathology results).

Methods

Immunohistochemical analysis of CEA. The immunohistochemical analysis of CEA expression was performed with a monoclonal antibody (anti-CEA clone A5B7) from Bio-optica (Milan, Italy) with hematoxylin as contrast staining for nuclei. Each analysis included a negative (using antirabbit serum instead of the monoclonal antibody against CEA) and a positive control, and each sample was examined by two independent pathologists. Samples in which more than 80% of the neoplastic cell were stained were considered positive.

Analysis of CEA and SOD mRNA. The acid guanidinium thiocyanate and phenol-clorophorm procedure was used to extract total RNA from biopsy samples and blood.[18] After extraction, RNA was spectrophotometrically quantitated at 260 nm (and at 280 nm to check the purity). The primers and the mixtures used for RT and PCR are described elsewhere,[13] starting from 500 ng of total RNA. The RT reaction was performed at 42°C for 30 minutes and the enzyme was then inactivated at 95°C for 15 seconds. The PCR conditions were as follows: a denaturation cycle at 94°C for 5 minutes, 35 cycles (95°C for 20 seconds, 65°C for 25 seconds, and 72°C for 20 seconds), and a final extension of 5 minutes at 72°C. The PCR product was analyzed both on acrylamide gel as previously described[15] and with a recently described dot-blot procedure.[19] Briefly, 4 μL of amplified DNA was spotted on a nitrocellulose filter and the filter was thus hybridized with a radiolabeled probe that had the following sequence: 5′-AGTGCTGGTTGGGGTTGCTC, which specifically hybridizes CEA cDNA. After hybridization, the filter was washed at 42°C for 15 minutes. RT-PCR followed by agarose electrophoresis was performed as previously reported.[15]

The amplified CEA cDNA was sequenced using the classical Sanger procedure both with an automated and with a manual procedure. In the automated procedure, the four termination reactions were marked with fluorescent didesoxynucleotides and the analysis of the fragments was performed using the 373A apparatus (Applied Biosystem, Perkin Elmer, Foster City, CA). The manual sequence was performed using the Sequenase kit, USB, Amersham (Cleveland, OH).

Analysis of serum CEA. Serum CEA was analyzed with a solid-phase radioimmunometric procedure, using the ELSA-2 CEA kit (Cis Biointernational, Février, France). The analytic sensitivity of this procedure is 0.3 ng/mL and the reference values in normal subjects are from 0 to 10 ng/mL.

Statistics. The Kolmogorov-Smirnov test was used to assess the distribution of serum CEA levels in the two populations.[20] Univariate statistical analysis was performed using the nonparametric Mann-Whitney *U* test to compare the distribution of CEA in the various groups of patients.[20] Diagnostic sensitivity and specificity were calculated according to Galen and Gambino.[21]

RESULTS

The analytic sensitivity of the RT-PCR dot-blot method for CEA mRNA analysis was evaluated testing scalar dilutions of RNA extracted from lung cancer cells with total RNA extracted from normal blood cells. The procedure detects CEA mRNA diluted as much as $1:10^6$ (Fig 1). The same analytic sensitivity was obtained with the original procedure based on RT-PCR followed by acrylamide gel electrophoresis.[15] The dot-blot procedure is highly specific, because the amplified DNA is revealed by hybridization with a complementary probe, and is reproducible. The sequence analysis performed on both blood and lung cancer tissue confirmed that the amplified product was CEA (Fig 2). All the samples of blood and lung cancer tissue were analyzed double-blind twice for CEA mRNA by two different operators. The data of the two sets of analyses were identical in all instances.

We next analyzed CEA mRNA in blood from the two

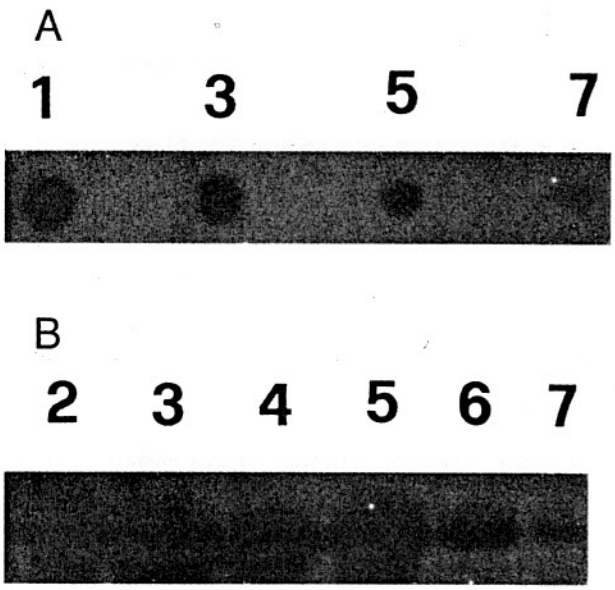

Fig 1. Sensitivity of CEA mRNA analysis by (A) RT-PCR dot-blot and (B) acrylamide gel electrophoresis. 1, Lung cancer tissue; 3, 1:100 dilution (RNA from lung cancer tissue: RNA from normal blood cells); 5, 1:10,000 dilution; 7, 1:1,000,000 dilution; 2, 4, and 6 intermediate dilutions.

groups of lung cancer patients (with and without distant metastases) and in the control groups of nonneoplastic and healthy individuals. The RT-PCR analysis for SOD mRNA, performed as a control at the same time as the CEA mRNA analysis, was positive in all subjects. The result of the analysis was negative in the two control groups. The result was positive (diagnostic sensitivity, 80%) in eight of 10 lung cancer patients with evidence of distant metastases (Table 1). An example of a positive result is shown in Fig 3. The two patients with negative

Table 1. CEA Expression in Lung Cancer Patients With Distant Metastases

Patient No.	Serum CEA (ng/mL)	CEA Expression (IHC, tissue)	CEA mRNA (RT-PCR, blood)	Stage	Histotype
1	22.7	Positive	Positive	IV	SQLC
2	3.4	Positive	Positive	IV	ADC
3	1.5	Negative	Negative	IV	SCLC
4	84.0	Positive	Positive	IV	SCLC
5	18.6	Positive	Positive	IV	SCLC
6	3.8	Positive	Positive	IV	SQLC
7	16.0	Positive	Positive	IV	ADC
8	25.1	Positive	Positive	IV	SQLC
9	24.0	Positive	Positive	IV	ADC
10	9.4	Negative	Negative	IV	ADC

Abbreviations: IHC, immunohistochemistry; SQLC, squamous cell lung cancer; ADC, adenocarcinoma; SCLC, small-cell lung cancer.

results were also negative at the immunohistochemical analysis for CEA in lung cancer tissue, which suggests that the cancer cells did not express CEA mRNA. Consequently, the low levels of circulating CEA protein in these patients could derive from sources other than lung tissue.

The immunohistochemical analysis for CEA performed on lung biopsies from the neoplastic tissue was negative in two of 14 patients affected by lung cancer without evidence of metastases (Table 2); all 12 samples of lung cancer tissue were positive when analyzed with RT-PCR analysis for CEA mRNA, which demonstrates the higher sensitivity of this approach. Four of 12 patients were positive for CEA mRNA in circulating cells; two of these patients (patients no. 1 and no. 8) developed bone metastases within 6 months of the analysis; of the other two, one died and one was lost to follow-up evaluation.

There were no significant differences in the concentrations of serum CEA in the two populations of cancer patients (mean values, 12.7 ng/mL in lung cancer with

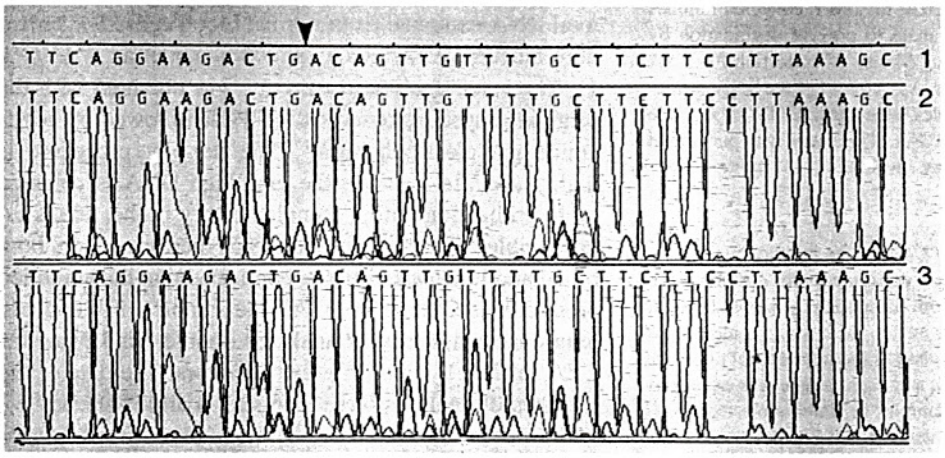

Fig 2. Sequence of CEA from an RT-PCR product extracted from circulating cells in a patient with metastatic lung cancer (2) and from lung cancer tissue obtained after bronchoscopy (3). 1, Sequence of the CEA.[22] Arrow indicates border between exons 8 and 9.

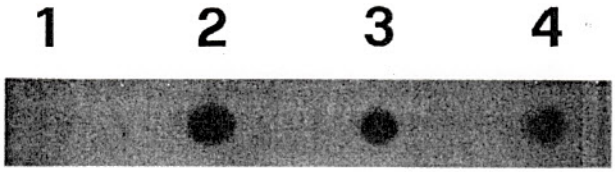

Fig 3. RT-PCR dot-blot procedure. Analysis of CEA mRNA from circulating cells. 1, negative control (blood from healthy subject); 2, positive control (lung cancer tissue); 3 and 4, blood from metastasized lung cancer.

no distant metastases *v* 20.8 ng/mL in patients with lung cancer with distant metastases). There was no correlation between serum CEA concentrations and the presence of circulating cells that expressed CEA mRNA.

DISCUSSION

The procedure for CEA mRNA analysis used in this study is highly efficient. Its analytic sensitivity is comparable to that obtained with the original RT-PCR method[15] and to that obtained by Gerhard et al,[13] who used the CEA mRNA analysis to detect bone metastatic cells from colonic cancer patients. The RT-PCR procedure followed by agarose gel electrophoresis described by Katz et al[7,9] for PSA mRNA analysis in circulating cells to detect micrometastases from prostatic carcinoma is 10-fold lower than that obtained with our method. The RT procedure followed by a nested PCR has high analytic sensitivity[10,11]; however, a high rate of false-positive results has been obtained with nested PCR amplification,[11] a finding supported by our experience with CEA and the PSA mRNA analysis.[22]

The improved dot-blot method is based on hybridization with a probe complementary to the amplified DNA, and thus the procedure is more specific than methods based on RT-PCR acrylamide electrophoresis[15] or agarose electrophoresis,[7,9] in which the PCR product is visualized as an electrophoretic band. Sequence analysis of the amplified cDNA confirmed the sequence of CEA.[23] The dot-blot procedure is rapid and easy to perform, and can be easily automated with the use of a robotic workstation,[19] with a subsequent reduction in costs.

The analysis of CEA mRNA in circulating cells had a satisfactory diagnostic sensitivity (80%) in identifying patients who have lung cancer with distant metastases. In our series, we had two false-negative cases; both of these cases were also negative at the immunohistochemical analysis for CEA in biopsy samples from the neoplasias. It is possible that in both cases, the low amount of CEA circulating as protein had been produced from sources other than pulmonary tissue. We did not analyze CEA mRNA in biopsy samples from patients who have lung cancer with distant metastases (to avoid a new biopsy sample) and thus we can only speculate that these neoplasias did not produce CEA. To increase the diagnostic sensitivity of RT-PCR analysis in detecting circulating metastatic cells, other mRNAs specifically expressed by lung cancer cells should be analyzed.

As far as we are aware, this is the first time RT-PCR has been used to detect metastatic cells in blood from lung cancer patients. The diagnostic sensitivity of PSA mRNA analysis in detecting micrometastases from prostate cancer in circulating blood cells is between 31%[8] and 75%.[7,9] We have recently obtained a diagnostic sensitivity of 50% with the PSA mRNA analysis.[22] The diagnostic sensitivity of albumin mRNA analysis in detecting micrometastases from hepatocarcinoma is 43%.[12] The scarce diagnostic sensitivity reported for mRNA analysis in detecting liver and prostatic cancer micrometastatic cells could depend on the migration of cancer cells in the buffy coat during the isolation procedures, a scarce number of circulating metastatic cells, or a poor analytic sensitivity of the methods.[24] In fact, a higher diagnostic sensitivity has been reported for cytometry as compared with PSA mRNA analysis by RT-PCR in identifying circulating metastatic cells in prostatic carcinoma.[10] However, the cytometric procedure has a low specificity because of the presence of circulating macrophages that bear PSA on the membrane.[10,25] In addition, cytometry is complex and cannot be automated.[13,25]

The scarce sensitivity of PSA and albumin mRNA analysis could be related to the fact that the metastatic circulating cells are usually poorly differentiated, while albumin and

Table 2. CEA Expression in Nonmetastatic Lung Cancer Patients

Patient No.	Serum CEA (ng/mL)	CEA (tissue) IHC	CEA (tissue) RT-PCR	CEA mRNA (RT-PCR, blood)	Stage	Histotype
1	3.7	+	+	+*	IIIB	ADC
2	2.6	−	+	−	I	SQLC
3	1.5	−	+	−	IIIB	SQLC
4	16.0	+	+	+†	I	ADC
5	1.8	+	+	−	IIIB	SQLC
6	2.6	+	+	−	IIIB	ADC
7	2.0	+	+	−	IIIA	SQLC
8	114.1	+	NT	+*	IIIB	ADC
9	0.9	+	NT	−	IIIB	SQLC
10	0.8	+	NT	−	IIIB	SQLC
11	3.6	+	NT	−	IIIB	ADC
12	2.7	+	NT	+†	IIIB	SQLC
13	2.6	+	NT	−	IIIB	SQLC
14	3.7	+	NT	−	IIIB	SQLC

Abbreviation: NT, not tested.
*Developed metastases within 6 months.
†Died or lost to follow-up.

PSA are usually expressed by well-differentiated cells. The higher diagnostic sensitivity obtained with CEA mRNA in lung cancer could be because CEA is expressed by scarcely differentiated cells. In any event, to avoid a high rate of false-negative results, the RT-PCR analysis could be performed first on neoplastic tissue when histology is performed, to verify the expression of the specific mRNA by cancer cells, and in cases of positive results, the procedure can be used to look for micrometastases in the blood.

No false-positive results were obtained among the eight normal controls and the eight nonneoplastic patients examined, while four patients who had lung cancer with no evidence of distant metastases were positive at the analysis. Two of these patients developed distant metastases within a few months. Thus, the positive CEA mRNA analysis on circulating cells could have been an early signal of the metastatic process. However, a larger population of nonmetastatic lung cancer patients should be analyzed and monitored before any conclusion is drawn on this point.

The role of mRNA analysis in detecting circulating micrometastases is promising, also in view of the poor performances of instrumental approaches. The analytic sensitivity of instrumental techniques, and thus the diagnostic sensitivity of these procedures in recognizing distant metastases from lung cancer, has increased over the last few years, but the number of false-positive or undefined diagnoses is high. Some investigators reported a 28% rate of indeterminant results,[26] and others a 40% rate of false-positive diagnoses of bone metastases from lung cancer obtained with radionuclide bone scan.[3] Ultrasound scanning to detect liver metastases gave a high rate of false-positive results, and ultrasound analysis of adrenal metastases a number of false-positive results due to the difficulty in distinguishing hyperplastic processes from metastases.[3] Nuclear magnetic resonance seems to be more specific in identifying adrenal metastases from lung cancer, but the procedure is expensive and difficult to use for screening purposes.

Thus, at present, given the high cost of instrumental procedures and the need to perform invasive procedures to confirm the presence of distant metastases, the tendency is to use instrumental procedures to search for distant metastases (particularly brain and abdominal) from lung cancer only in patients who show clinical signs and symptoms.[4] This strategy obviously reduces the possibility of early diagnosis, because clinical and laboratory data are not sensitive in identifying metastases in lung cancer patients and because approximately 50% of patients with distant metastases are clinically asymptomatic.[4] In this context, the simple and inexpensive procedure for the early identification of metastases from lung cancer described here could be useful and could reduce the number of invasive procedures and needless surgical operations.

In conclusion, the RT-PCR dot-blot procedure shows a high analytic sensitivity and specificity for CEA mRNA analysis, and CEA mRNA analysis from blood samples is a sensitive (80%) method for the identification of lung cancer patients with distant metastases that could contribute to the early identification of circulating cells from lung cancer.

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