Detection of Circulating Tumor Cells in Carcinoma Patients by a Novel Epidermal Growth Factor Receptor Reverse Transcription-PCR Assay[1]

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ABSTRACT

The epidermal growth factor receptor (EGFR) is overexpressed in 50–70% of human primary breast, lung, and colon carcinomas, whereas it is not usually expressed in hematopoietic cells. We developed a novel reverse transcription-PCR (RT-PCR)-Southern blot assay for the detection of circulating, EGFR mRNA-expressing tumor cells in carcinoma patients. The assay was set up by increasing the amount of cDNA step by step in the PCR reaction. The highest sensitivity and specificity were found when using 800 ng of cDNA in the PCR reaction. Peripheral blood samples from 91 patients with either colon (38), lung (30), or breast (23) carcinomas and from 38 healthy volunteers were analyzed. EGFR transcripts were found in 44 of 75 (59%) patients with metastatic carcinoma and in 4 of 38 (10.5%) healthy donors ($P < 0.001$; χ^2 test). The expression of EGFR, cytokeratin 19, and carcinoembryonic antigen mRNA in blood samples from patients with metastatic colon carcinoma was compared. EGFR, cytokeratin 19, and carcinoembryonic antigen transcripts were found in 8 of 11 (73%), 3 of 11 (27%), and 5 of 11 (45%) patients, respectively. Furthermore, two of seven (29%) Dukes' B and five of nine (55%) Dukes' C colon carcinoma patients were found to express EGFR mRNA in the peripheral blood. All patients that expressed EGFR transcripts in the peripheral blood were found to express the EGFR protein in the corresponding primary carcinoma, as assessed by immunohistochemistry. These data suggest that the EGFR assay that we developed is a highly specific and sensitive technique to detect circulating tumor cells in patients affected by different carcinoma types.

INTRODUCTION

Metastatic spreading through blood vessels is the most important factor affecting the prognosis of patients with primary carcinomas. Patients with primary cancer such as breast, colon, or lung carcinomas who have undergone radical, curative surgery have a recurrence rate as high as 20–60%. Lymph node involvement is the most important prognostic factor for tumor recurrence in these patients. However, 30–50% of carcinoma patients who show no evidence of disease in the locoregional lymph nodes will have a recurrence at a distant site. Therefore, novel prognostic factors that separate patients into low-risk and high-risk groups in terms of recurrence and need for adjuvant therapy are required.

The metastatic process is a complex cascade of events: tumor cells in the primary site must erode the basement membrane; penetrate a blood vessel; and spread to distant sites (1). In this regard, detection of carcinoma cells in the blood could be important to identify carcinoma patients at high risk of relapse. In fact, immunochemical detection of micrometastases in the bone marrow of breast, colon, and gastric carcinoma patients has been shown to correlate with early disease relapse (2–4). More recently, the RT-PCR[3] has been proposed as a rapid screening assay to detect systemic tumor cell dissemination in peripheral blood and/or bone marrow. RT-PCR shows a 10–100-fold higher sensitivity than routine immunocytochemistry methods. Several markers have been used to detect circulating cancer cells. In particular, CKs and CEA have been proposed as markers to detect circulating tumor cells in gastrointestinal, breast, and/or lung carcinoma patients (5–8). Few reports have suggested that a correlation between expression of these markers in the peripheral blood and/or in the bone marrow and patient outcome might exist (6, 9–12).

The EGFR is expressed in all cell types with the exception of hematopoietic cells. More importantly, the EGFR has been found to be overexpressed in 50–70% of human primary colon, lung, and breast carcinomas as well as in other tumor types (13). Therefore, the EGFR might represent a suitable marker for detection of circulating tumor cells in patients affected by different carcinoma types. In this context, Mapara *et al.* (14) developed an EGFR RT-PCR assay to detect tumor cells in

Received 8/12/99; revised 1/4/00; accepted 1/6/00.
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[1] Supported by a grant from the Associazione Italiana per la Ricerca sul Cancro (to N. N.).
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[3] The abbreviations used are: RT-PCR, reverse transcription-PCR; EGFR, epidermal growth factor receptor; CK, cytokeratin; CEA, carcinoembryonic antigen.

leukapheresis products obtained from breast cancer patients. However, this assay used a single-step RT-PCR, and it was less sensitive than immunocytochemical detection of tumor cells. More recently, a nested PCR assay for detection of EGFR mRNA has been developed (15). This assay also showed a low sensitivity because EGFR transcripts were found in only 22% of peripheral blood samples from metastatic breast cancer patients.

The aim of this study was to develop a novel, nested RT-PCR-Southern blot assay for the detection of EGFR mRNA in peripheral blood. For this purpose, we analyzed peripheral blood samples from healthy volunteers and from breast, colon, and lung carcinoma patients. Finally, we compared the sensitivity and specificity of this technique with those of other previously reported RT-PCR assays.

MATERIALS AND METHODS

Patients. Ninety-one consecutive patients with either colon, lung, or breast carcinomas admitted at our institute between July 1998 and July 1999 were examined (Tables 2 and 4). The only exclusion criterion was previous treatment. Thirty-eight healthy volunteers were also analyzed as controls. After obtaining informed consent from patients, peripheral blood samples (5 ml) were obtained by aspiration into EDTA-containing syringes.

Cell Lines. The human colon carcinoma GEO cell line and breast carcinoma MDA-MB-468 cell line were grown as described previously (16, 17).

RNA Preparation. Five-ml aliquots of peripheral blood were processed within 1 h of being obtained from the patient. The blood samples were mixed with 1 ml of 5% dextran-saline solution and left to set for 30 min at room temperature to yield erythrocyte sediment. Supernatant was collected and centrifuged at 500 × *g* for 10 min at 4°C. The cells were then suspended in 1 ml of nucleic acid extraction buffer and frozen at −70°C until RNA extraction was performed. Total cellular RNA was obtained by the acid guanidine isothyocianate-phenol-chloroform extraction procedure, as described previously (18).

PCR Primers and RT-PCR. EGFR primers for the nested PCR were designed from the sequences of the EGFR human gene and cDNA. In the first-round PCR, we used the EGFR primers published by Mapara *et al.* (14): primer A, 5′-TCTCAGCAACATGTCGATGG-3′, which corresponds to sequence 702–721 of primer B, EGFR cDNA; and primer B, 5′-TCGCACTTCTTACACTTGCG-3′ (amino acids 1156–1175). These primers amplify a 473-bp fragment of the EGFR cDNA. The nested PCR was performed by using primer A and primer C (5′-TCACATCCATCTGGTACGTG-3′; amino acids 1005–1024), and the PCR product was 322 bp in length. The RT reaction was performed using 2-μg aliquots of total cellular RNA. First-strand cDNA synthesis was carried out in a reaction volume of 20 μl using the RNA synthesis kit of Perkin-Elmer (Branchburg, NJ) as suggested by the manufacturer. An 8-μl aliquot of this reaction was subsequently used for EGFR PCR amplification in a 25-μl reaction buffer containing 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, the four deoxynucleoside triphosphates (0.25 mM each), 10 pm of each primer (A and B), and 0.5 unit of Taq Gold polymerase (Perkin-Elmer). Samples for PCR amplification were prepared in a laminar flow hood to avoid contamination. PCR was performed for 30 cycles consisting of 5 cycles of 30 s at 94°C, 45 s at 60°C, and 45 s at 72°C and 25 cycles of 30 s at 94°C, 45 s at 55°C, and 45 s at 72°C in a GeneAmp PCR System 9700 (Perkin-Elmer). The samples were heated for 10 min at 94°C before the first cycle, and the extension was lengthened to 10 min during the last cycle. One μl of the first-round PCR product was used for the nested PCR. The PCR conditions for the nested PCR reaction were similar to those for the first-round PCR, with the following exceptions: (*a*) primers A and C were used for amplification; and (*b*) the total PCR number of cycles was 35 (5 + 30 cycles). Fifteen μl of the PCR product were electrophoresed through a 1.5% agarose gel. The gel was stained with ethidium bromide to allow visualization of the DNA, which was then denatured and transferred to a nylon membrane. Finally, the membrane was hybridized with an EGFR cDNA probe. The cDNA product was visualized using streptavidin-alkaline phosphatase-coupled enhanced chemiluminescence (New England Biolabs, Beverly, MA).

The GAPDH PCR reaction was performed as described previously (19). CEA and CK19 nested PCR-Southern blot assays were performed as described previously (5, 7), with the following exceptions: (*a*) the PCR reaction was carried out in a 25-μl volume; (*b*) a hot start technique with TaqGold was used; and (*c*) different amounts of cDNA were used, as detailed in "Results."

Analysis of each sample was repeated at least two times. Two identical, consecutive results completed testing. A positive control and several negative controls were included in each experiment.

Immunohistochemistry. EGFR expression in formalin-fixed, paraffin-embedded tissues was assessed as described previously (20) by using the C216 anti-EGFR monoclonal antibody (DBA, Milan, Italy).

RESULTS

We used RNA from EGFR-positive tumor cell lines to set up the EGFR RT-PCR-Southern blot assay. In particular, we analyzed EGFR expression in GEO cells, human colon carcinoma cells that express 3×10^4 EGFR sites/cell, and in MDA-MB-468 human breast carcinoma cells, which express 3×10^6 EGFR sites/cell (16, 17). We evaluated the sensitivity of the assay by using limiting dilution of mRNA from GEO and MDA-MB-468 cells. The sensitivity of the assay was correlated with the level of expression of the EGFR in the carcinoma cells. In fact, we were able to detect a specific EGFR transcript in 3 pg of GEO cell total RNA and in 30 fg of MDA-MB-468 total RNA by using the RT-PCR technique followed by Southern blot (Fig. 1; data not shown). We have previously found that a high *in vitro* sensitivity does not always correspond to high sensitivity in the patients (19). Therefore, we further developed the assay by contemporary analysis of peripheral blood samples obtained from a small group of colon carcinoma patients and healthy volunteers (Table 1). In particular, we increased the amount of cDNA step by step in the first PCR reaction to increase the sensitivity of the assay. We detected EGFR mRNA in 5 of 11 (45%) colon carcinoma patients and in none of the healthy donors by using 500 ng of cDNA in the first PCR reaction. When we increased the amount of cDNA up to 800 ng in the first-round PCR, EGFR transcripts were observed in 8 of 11 (73%) colon carcinomas and in 1 of 10 (10%) healthy

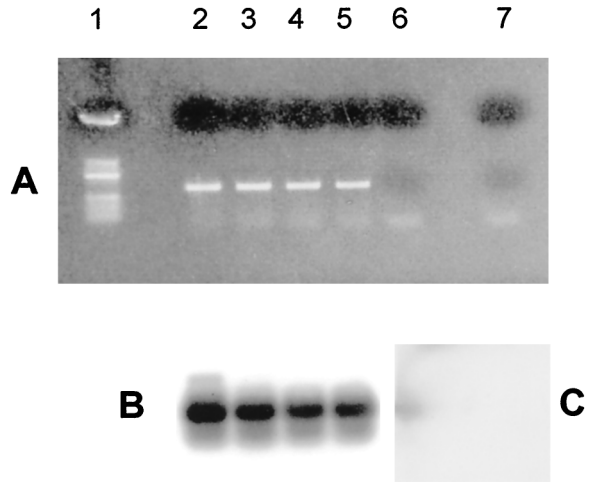

Fig. 1 EGFR mRNA expression in GEO cells. *Lane 1*, marker (50-bp ladder, Boehringer Mannheim, Mannheim, Germany); *Lane 2*, 30 ng of GEO cDNA; *Lane 3*, 3 ng; *Lane 4*, 300 pg; *Lane 5*, 30 pg; *Lane 6*, 3 pg; *Lane 7*, negative control. *A*, ethidium bromide staining of the gel; *B*, Southern blot analysis. The filter was exposed for 5 s. Thereafter, the filter was cut, and the right part was exposed for 1 min (*C*).

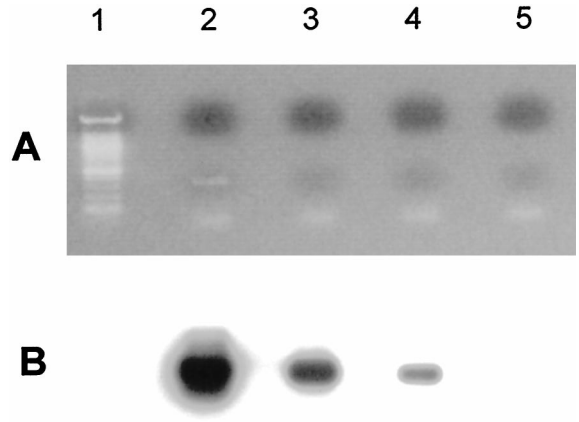

Fig. 2 Sensitivity of EGFR mRNA analysis. *Lane 1*, marker (100-bp ladder, Boehringer Mannheim); *Lane 2*, $1:10^3$ dilution of RNA from GEO cells with RNA from normal leukocytes; *Lane 3*, $1:10^4$ dilution; *Lane 4*, $1:10^5$ dilution; *Lane 5*, $1:10^6$ dilution. *A*, ethidium bromide staining of the gel; *B*, Southern blot analysis.

Table 1 Analysis of EGFR expression in peripheral blood samples from patients with metastatic colon carcinoma and from healthy donors

	Colon carcinomas	Healthy donors
Total cDNA (ng)	Positive/total (%)	Positive/total (%)
500	5/11 (45)	0/10 (0)
800	8/11 (73)	1/10 (10)

Table 2 Analysis of EGFR expression in peripheral blood samples from patients with metastatic lung, colon, or breast carcinoma and from healthy donors

Carcinoma	Stage	Positive samples (%)	*P vs.* Controls[a]
Lung	IV	17/30 (57)	<0.001
Breast	IV	11/23 (48)	0.003
Colon	Dukes' D	16/22 (73)	<0.001
Total		44/75 (59)	<0.001
Controls		4/38 (10.5)	

[a] χ^2 test.

volunteers. The latter PCR conditions were used in the following experiments.

The sensitivity of the RT-PCR-Southern blot assay for detection of EGFR mRNA was also evaluated by testing a serial dilution of RNA extracted from GEO cells with total RNA extracted from normal blood cells. The method was able to detect EGFR transcripts in GEO cell total RNA diluted as much as $1:10^5$, which corresponds to 8 pg of GEO cell RNA diluted in 800 ng of total RNA from normal leukocytes (Fig. 2).

We next analyzed EGFR mRNA expression in 75 patients with disseminated colon, lung, or breast carcinomas and in 38 healthy volunteers (Table 2). All of the samples showed a positive GAPDH RT-PCR signal, indicating the presence of intact RNA and successful first-strand cDNA preparation (data not shown). We detected EGFR transcripts in 44 of 75 (59%) carcinoma patients and in 4 of 38 (10.5%) healthy donors (Fig. 3; Table 2). This difference was statistically significant ($P < 0.001$, χ^2 test). The highest sensitivity was observed in colon carcinoma (73%), whereas only 48% of the breast carcinoma patients were found to be positive. However, this difference was not statistically significant as assessed by the χ^2 test. Southern blot analysis of the PCR product confirmed the specificity of the reaction, and it also increased the number of positive patients. In fact, an EGFR band was evident after ethidium bromide staining of the gel in approximately two-thirds of the positive samples (Fig. 3*A*). An additional 35% of samples were found to be positive after Southern blot analysis (Fig. 3*B*).

We compared the sensitivity and specificity of the EGFR with other markers that have been used for detection of circulating tumor cells, such as CEA and CK19. In particular, we analyzed the expression of CEA and CK19 transcripts in peripheral blood samples from patients with metastatic colon carcinomas (Dukes' D stage) that had been previously screened for EGFR expression. We used a nested PCR technique followed by Southern blot analysis for both markers, as described previously (5, 7). A small group of healthy donors was also analyzed to assess the specificity of these different techniques. CEA was found to be expressed in 5 of 11 (45%) colon carcinomas and in 2 of 10 (20%) healthy donors when 100 ng of cDNA were used in the first PCR reaction (Table 3). Three of 11 (27%) colon carcinoma patients and 1 of 10 (10%) healthy donors were positive for CK19 expression when 200 ng of cDNA were used (Table 3). As well as we did for EGFR, we tried to improve the sensitivity of the technique by increasing the amount of cDNA in the first PCR reaction. However, this also resulted in a decrease of the specificity of both the CEA and CK19 assays (Table 3).

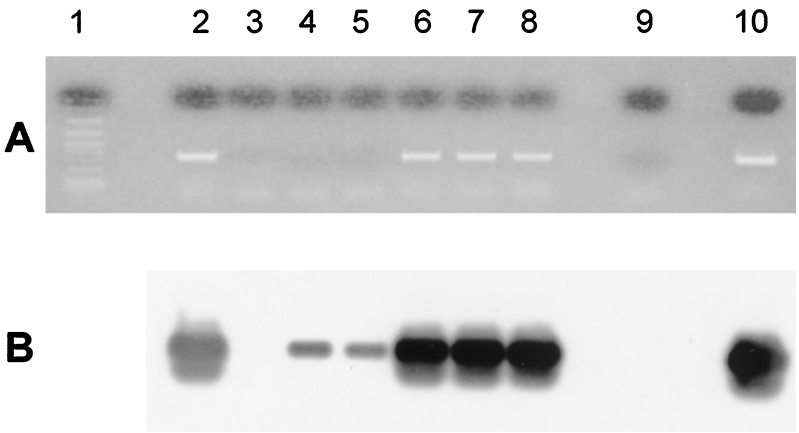

Fig. 3 Analysis of EGFR expression in peripheral blood samples from patients with metastatic colon carcinoma. *Lane 1*, marker (Amplisize DNA Standard; Bio-Rad Laboratories, Milan, Italy); *Lanes 2–8*, peripheral blood samples from colon carcinoma patients; *Lane 9*, negative control (blood from healthy volunteer); *Lane 10*, positive control (30 pg of GEO cDNA).

Table 3 Analysis of EGFR, CEA, and CK19 expression in peripheral blood samples from patients with metastatic colon carcinoma and from healthy donors

	Colon carcinomas	Healthy donors
Marker (Total cDNA)	Positive/total (%)	Positive/total (%)
EGFR (800 ng)	8/11 (73)	1/10 (10)
CEA (100 ng)	5/11 (45)	2/10 (20)
CEA (500 ng)	10/11 (91)	6/10 (60)
CK19 (200 ng)	3/11 (27)	1/10 (10)
CK19 (500 ng)	6/11 (55)	8/10 (80)

Table 4 Analysis of EGFR expression in peripheral blood samples from colon carcinoma patients with different stages of disease

Stage	Positive samples (%)
Dukes' B	2/7 (29)
Dukes' C	5/9 (55)
Dukes' D	16/22 (73)

We next assessed the expression of EGFR transcripts in a small group ($n = 16$) of colon carcinoma patients with resectable disease. We detected EGFR mRNA in two of seven (29%) Dukes' B patients and in five of nine (55%) Dukes' C patients (Table 4). Finally, we analyzed by immunohistochemistry the expression of EGFR in the primary tumors of 10 patients with either Dukes' B or Dukes' C colon carcinoma (Table 5). All patients who expressed EGFR transcripts in the peripheral blood were found to express the EGFR protein in the corresponding primary carcinoma. The expression of the EGFR transcripts in the primary tumors was also confirmed by RT-PCR analysis of the RNA extracted from the same formalin-fixed, paraffin-embedded tissues that were used for immunohistochemical analysis (data not shown). We assessed EGFR expression in tumors from two Dukes' D patients (patients 104 and 110; Table 5). In fact, both patients underwent surgery because distant metastases were not detected by routine staging procedures. However, liver metastases were found in both cases during surgery. The tumors from the two patients were both positive for EGFR expression, and the peripheral blood samples from these patients were both found to express EGFR transcripts (Table 5).

Table 5 Analysis of EGFR expression in peripheral blood samples and in primary tumors from colon carcinoma patients

Stage	Peripheral blood	Tumor
(patient no.)	EGFR PCR	EGFR ICC
B (94)	+	70 (++)[a]
B (98)	−	85 (++)
B (111)	−	0
B (112)	−	30 (+)
C (96)	+	40 (++)
C (97)	−	0
C (99)	+	30 (+)
C (100)	−	50 (+)
C (102)	+	80 (+++)
C (103)	+	80 (+++)
D (104)	+	50 (+)
D (110)	+	20 (+)

[a] Percentage of EGFR-positive tumor cells (intensity of staining).

DISCUSSION

In this study, we describe a novel EGFR RT-PCR-Southern blot assay for the detection of circulating tumor cells in patients affected by different carcinoma types. This assay is highly sensitive and specific. In this regard, we were able to detect EGFR mRNA from GEO colon carcinoma cells diluted as much as $1:10^5$ with RNA from normal blood cells. Other previously published RT-PCR methods were more sensitive because specific transcripts were detected at up to a $1:10^6$ or $1:10^7$ dilution (5–7). This difference might be due to the fact that we chose a cell line that expresses low levels of EGFR binding sites (3×10^4 binding sites/cell) and mRNA to assess the lowest sensitivity of the assay for such experiments. However, we have previously found that very sensitive *in vitro* methods may show a very low sensitivity in the patients (19). For this reason, we set up the EGFR RT-PCR assay by using increasing concentrations of cDNA from carcinoma patients and healthy donors. A nested PCR assay for detection of EGFR mRNA has been recently developed by Leitzel *et al.* (15). This assay is capable of detecting a lower limit of 100 fg of total RNA from A431 cells, which overexpress the EGFR. The *in vitro* sensitivity of this assay is similar to that of our technique. In fact, we could detect EGFR transcripts in 30 fg of RNA from MDA-MB-468 cells,

which express levels of EGFR similar to A431 cells (data not shown). The sensitivity of our assay in the patients was much higher because Leitzel *et al.* (15) only found EGFR transcripts in 22% of metastatic breast carcinoma patients. Breast cancer patients also showed the lowest percentage of positive samples in our study (48%). Furthermore, differences in blood sampling and processing, RNA extraction, and Southern blot analysis might account for the higher sensitivity of our technique in the patients. In this regard, no increase in the sensitivity of the RT-PCR was observed by Leitzel *et al.* (15) by using Southern blot analysis, whereas Southern blot increased the sensitivity of our assay by 50%.

The higher *in vivo* sensitivity of our assay might also justify the fact that we found EGFR transcripts in 4 of 38 (10.5%) healthy donors, whereas Leitzel *et al.* (15) found no expression of EGFR mRNA in 23 control individuals. None of the volunteers who were screened in this study had clinical evidence of tumor disease at the time of blood withdrawal. It has long been established that the EGFR is not usually expressed in hematopoietic cells (13). It is conceivable that our technique is able to detect illegitimate transcription of the EGFR gene in leukocytes (21). However, the specificity of our EGFR assay was confirmed by the observation that no false positives were found in a small group of colon carcinoma patients in whom the expression of the EGFR protein in the tumor was assessed. In fact, all patients who were positive for EGFR mRNA expression in the blood showed detectable levels of EGFR protein in the primary tumor, whereas none of the patients with tumors negative for EGFR expression was found to express EGFR transcripts in the peripheral blood. In this context, no correlation was found between the intensity of EGFR staining in the tumor and the quantitative results of the RT-PCR assay on peripheral blood. The ability of the RT-PCR assay to detect EGFR transcripts is clearly correlated to both the level of expression of the EGFR and the number of tumor cells that are circulating. These two parameters are not necessarily correlated. The high sensitivity of our assay also allowed us to detect EGFR mRNA in approximately 45% (7 of 16) of colon carcinoma patients with resectable disease, whereas Leitzel *et al.* (15) failed to demonstrate the presence of EGFR transcripts in locally recurrent breast cancer patients and in adjuvant breast cancer patients. Clinical follow-up of patients with localized, resectable colon carcinoma has been started at our institution to assess whether patients at high risk of relapse might be recognized by using this assay.

Our data suggest that the background level of expression of EGFR in leukocytes is lower when compared to other markers, such as CEA and CK19. In fact, a lower number of false positives for EGFR expression were seen in a group of healthy donors, when compared with CEA and CK-19 (Table 3). In particular, CK19 showed a very low specificity. Several reports have suggested that illegitimate transcription of CK19 mRNA occurs in human leukocytes (22, 23). The use of a nested PCR technique often results in detection of CK19 transcripts in healthy volunteers (24). Expression of CEA mRNA has been described recently in 76–80% of peripheral blood samples from patients with metastatic carcinomas by using a nested PCR or a PCR dot-blot technique, respectively (6, 9). A significant correlation has been found between expression of CEA mRNA and tumor recurrence in breast and gastrointestinal carcinoma patients by using a nested PCR approach (9). Several reports have also demonstrated that a high percentage of false positive results occurs when using a nested PCR analysis for CEA mRNA expression (23, 25). In our hands, the nested PCR CEA assay showed a higher specificity when compared with CK19. However, when we tried to increase its sensitivity by increasing the amount of cDNA in the PCR reaction, a high number of false positive results were observed.

These results suggest that the EGFR might represent a suitable marker for detection of circulating tumor cells. In this regard, this study is the first to demonstrate that circulating tumor cells can be detected in the peripheral blood of patients with lung or colon carcinoma. However, the EGFR is expressed in several human carcinoma types other than breast, lung, and colon tumors in which this assay could be used (13). Several pieces of experimental evidence suggest that the EGFR is involved in tumor formation and progression. Overexpression of EGFR has been correlated with prognosis and metastatic spreading in several carcinoma types, such as breast, head and neck, and lung malignancies (13). In this context, EGFR expression might be more informative on patient's prognosis than CK or CEA expression because of its involvement in the pathogenesis of human carcinomas. Finally, novel therapeutic approaches based on EGFR blocking antibodies, EGFR tyrosine kinase inhibitors, or antisense oligonucleotides directed against either EGFR or its ligands have been developed (26, 27). The EGFR assay could be used to monitor the response of the tumor to these novel agents by monitoring the levels of EGFR-positive cells shed by the carcinoma during therapy.

In conclusion, we believe that the EGFR assay that we have developed is a highly specific and sensitive technique to detect circulating carcinoma cells. Studies on a larger cohort of patients and in different carcinoma types are required to confirm these findings. More importantly, clinical follow-up of patients with resectable disease will allow us to assess whether high-risk patients can be selected by using this approach.

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