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Carcinoembryonic antigen mRNA analysis detects micrometastatic cells in blood from lung cancer patients

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Carcinoembryonic antigen mRNA analysis detects micrometastatic cells in blood from lung cancer patients. G. Castaldo, R. Tomaiuolo, A. Sanduzzi, A. Ponticiello, I. Marchetiello, F. Salvatore. ©ERS Journals Ltd 2003.

ABSTRACT: The current authors previously identified circulating cells expressing carcinoembryonic antigen (CEA) messenger ribonucleic acid (mRNA) in 80% of lung cancer patients bearing distant metastases. The current study prospectively validated the data on a novel cohort and extended the study to other mRNAs expressed by neoplastic cells.

CEA, cytokeratin 19 and 20, aldolase A and epithelial glycoprotein 2 (EPG2) mRNA was analysed by reverse transcriptase-polymerase chain reaction in circulating cells from 19 healthy controls, and in biopsies and blood at diagnosis from 32 lung cancer patients monitored for 24 months.

Aldolase A and cytokeratin 19 mRNA occurred in circulating cells of all controls; cytokeratin 20 was not expressed by any lung cancer biopsy. EPG2 mRNA occurred in all biopsies but not in the patients' circulating cells. CEA mRNA occurred in 29/32 (90.6%) biopsies and in 17/32 mRNA samples from circulating cells from lung cancer patients. Of these positive patients 12/17 developed metastases within 9 months of mRNA analysis. Three positive patients died, one was lost to follow-up, and one did not develop metastases within 24 months. Of the negative patients 12/15 did not develop metastases during the 24-month follow-up; one patient was lost to follow-up, one did not express CEA, and another developed metastases.

Unlike in other neoplasias, cytokeratin 19 and 20, aldolase A and epithelial glycoprotein 2 messenger ribonucleic acid are not useful for the detection of circulating cancer cells in lung cancer. Carcinoembryonic antigen messenger ribonucleic acid analysis in circulating cells helps to identify lung cancer patients at a greater risk of metastases.

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Lung cancer is the most frequent neoplasia in adult males and about one half of patients has distant metastases at diagnosis [1], or develops metastases within a few months of surgery [2]. The detection of metastases affects staging, prognosis and therapy in lung cancer [3], *i.e.* patients with a higher risk for metastatic relapse could benefit from adjuvant chemotherapy [4]. Immunocytochemistry has been used to detect neoplastic cells in blood from tumour-bearing patients. However, immunocytochemistry is complex, difficult to automate and gives false positive results because of non-specific antigen expression by macrophages [5]. It has been suggested that the more sensitive [6] reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of messenger ribonucleic acid (mRNA) expressed by neoplastic cells be used to detect tumour cells in blood in such human neoplasias as colon, pancreas and lung [7], prostate [8], and breast [6]. However, several mRNA species expressed by human neoplasias are illegitimately transcripted in normal blood cells [9, 10], and the existence of processed pseudogenes could lower the specificity of RT-PCR analysis [9, 11]. Furthermore, because gene expression is heterogeneous in human neoplasias, a single mRNA marker might not be sufficiently sensitive to detect circulating micrometastases [9].

In a retrospective study, the current authors found carcinoembryonic antigen (CEA) in circulating cells of >80% of lung cancer patients with distant metastases [12]. The current report studied CEA mRNA and four other mRNA species specifically expressed by human neoplasias in a novel, independent cohort of lung cancer patients, prospectively monitored for 24 months: cytokeratin 19 mRNA expressed by lung cancer and other tumour cells [5, 13], cytokeratin 20, detected in circulating cells from metastatic gastrointestinal patients by RT-PCR [7, 14, 15], aldolase A mRNA, which discriminates between cirrhosis and hepatocarcinoma [16], and epithelial glycoprotein 2 (EPG2) mRNA that has been used to detect circulating micrometastases from colon cancer [17].

Materials and methods

Patients and samples

Each patient gave informed consent to the study; the study met the approval criteria of the Ethics Committee of the Medical School (University of Naples "Federico II", Italy). The current study analysed: 1) blood samples from 19 healthy volunteers to exclude illegitimate transcription of mRNA species by circulating cells; 2) biopsies obtained by

fibrobronchoscopy (at the time of sampling for cytohistology) from 32 unselected lung cancer patients: 19 squamous cell carcinoma (stage Ia: 1; stage Ib: 5; stage IIb: 4; stage IIIa: 7; stage IV: 2); 13 adenocarcinoma (stage Ia: 1; stage Ib: 3; stage IIb: 2; stage IIIa: 6; stage IV: 1); 26 males and 6 females (mean age 61.2 yrs) to verify the expression of each mRNA species by neoplastic cells; the biopsies were divided into two macroscopically homogeneous samples, one for histology and the other for mRNA analysis; and 3) blood samples from the 32 lung cancer patients before treatment. The reference diagnosis for each patient was obtained by histological analysis of fibrobronchoscopic biopsies according to the 1999 modified World Health Organisation Criteria [18], and staging (table 1), based on total-body computed tomography (CT) and bone scintigraphy with technetium 99 m, and was expressed as the tumour-node-metastasis score [19]. The patients, treated by surgery (22 cases), and by chemotherapy (5 cases), were monitored every 3 months for 24 months with CT and bone scintigraphy. Two patients were lost to follow-up and three patients died within 3 months. Biopsies were collected in ethylene diamine tetra-acetic acid (EDTA) tubes at the time of sampling for routine laboratory analysis. To avoid contamination from skin epithelial cells potentially expressing target mRNA [20], blood was sampled by venipuncture and the EDTA tube was used last. Biopsies were collected in tubes containing guanidinium thiocyanate to prevent ribonucleic acid (RNA) degradation [12]. All samples were processed within 2 h of collection for RNA extraction, and then analysed by RT-PCR.

Methods

RNA was extracted, and CEA and aldolase A mRNA were analysed as previously described [12, 16]. Cytokeratin 19, cytokeratin 20 and EPG2 mRNA were analysed as follows: RT: 25°C for 10 min; 42°C for 30 min; 95°C for 5 min; polymerase chain reaction (PCR): 94°C for 5 min, followed by 30 cycles (95°C for 30 s, 55°C for 30 s, 72°C for 30 s) and 72°C for 5 min, using the previously described primers cytokeratin 19 [14], cytokeratin 20 [21] and EPG2 [17]. These primers were designed to exclude amplification of known pseudogenes. In addition, the two PCR primers excluded genomic deoxyribonucleic acid (DNA) amplification because they hybridise two sequential exons of the gene. Several amplified complimentary deoxyribonucleic acid (cDNA) samples obtained from colon and lung cancer tissue were sequenced using the classical Sanger method and an automated device [12] to verify the specificity of the RT-PCR reaction. Furthermore, to verify the absence of DNA contamination, each RNA sample was analysed twice (*i.e.* with and without reverse transcriptase).

Each analytical series included two positive and two negative (no mRNA) controls. To check RNA extraction and RT-PCR,

Table 1. – Carcinoembryonic antigen (CEA) and epithelial glycoprotein 2 (EPG 2) messenger ribonucleic acid expression in 32 lung cancer patients

Patient no.#	Histotype	TNM at diagnosis	Stage	Distant metastases (lead time to appearance)	CEA mRNA		EPG 2 mRNA expression	
					Biopsy	Blood	Biopsy	Blood
14	SQLC	T1 N0 M0	Ia	No metastases¶	+	-	+	-
16	ADC	T1 N0 M0	Ia	Lost to follow-up	+	-	+	-
3	SQLC	T2 N0 M0	Ib	No metastases	+	-	+	-
6	SQLC	T2 N0 M0	Ib	No metastases	-	-	+	-
12	SQLC	T2 N0 M0	Ib	No metastases	-	-	NT	-
13	ADC	T2 N0 M0	Ib	No metastases	+	-	+	-
15	SQLC	T2 N0 M0	Ib	No metastases	+	-	NT	-
17	ADC	T2 N0 M0	Ib	No metastases	+	-	+	-
26	SQLC	T3 N0 M0	IIb	No metastases	+	-	+	-
32	ADC	T3 N0 M0	IIb	No metastases	+	-	NT	NT
27	SQLC	T3 N1 M0	IIIa	No metastases	+	-	+	-
23	ADC	T3 N1 M0	IIIa	No metastases	+	-	+	-
9	ADC	T3 N1 M0	IIIa	9 months	+	-	+	-
22	SQLC	T3 N1 M0	IIIa	No metastases	+	-	+	-
28	SQLC	T3 N1 M1	IV	At diagnosis	-	-	+	-
10	ADC	T2 N2 M0	IIIa	6 months	+	+	+	-
11	ADC	T2 N2 M0	IIIa	6 months	+	+	+	-
31	SQLC	T2 N2 M0	IIIa	6 months	+	+	NT	NT
8	SQLC	T2 N0 M0	Ib	Lost to follow-up	+	+	+	-
24	ADC	T2 N0 M0	Ib	12 months	+	+	+	-
7	SQLC	T2 N1 M0	IIb	Died within 3 months	+	+	+	-
18	SQLC	T2 N1 M0	IIb	9 months	+	+	+	-
21	ADC	T2 N1 M0	IIb	No metastases	+	+	+	-
29	SQLC	T2 N1 M0	IIb	6 months	+	+	NT	NT
2	ADC	T3 N1 M0	IIIa	6 months	+	+	+	-
4	ADC	T3 N1 M0	IIIa	Died within 3 months	+	+	+	-
5	SQLC	T3 N1 M0	IIIa	6 months	+	+	+	-
20	SQLC	T3 N2 M0	IIIa	3 months	+	+	+	-
25	SQLC	T3 N2 M0	IIIa	9 months	+	+	+	-
30	SQLC	T3 N2 M0	IIIa	Died within 3 months	+	+	+	-
1	SQLC	T2 N2 M1	IV	At diagnosis	+	+	+	-
19	ADC	T3 N2 M1	IV	At diagnosis	+	+	+	-

#: listed according to staging; ¶: within 24 months of follow-up. TNM: tumour-node-metastasis; SQLC: squamous cell lung cancer; ADC: adenocarcinoma; NT: not tested; +: positive; -: negative.

all samples were analysed for glyceraldehyde 3-phosphate dehydrogenase and superoxide dismutase housekeeping mRNA [12]. To prevent carry-over contamination, the preparation of solutions, DNA extraction, RT-PCR, and the processing of PCR products were done in different rooms [6, 22]. Each sample was analysed twice by two independent operators, each unaware of the results obtained by the other.

Results

Sequence analysis, performed on four lung cancer samples for aldolase A, CEA, and EPG2 and on four colon carcinoma samples for cytokeratin 19 and cytokeratin 20, confirmed that the RT-PCR specifically identified the mRNA species under investigation. The preliminary analysis of blood samples from normal subjects showed that aldolase A and cytokeratin 19 were expressed by circulating blood cells of all 19 normal subjects, whereas CEA, EPG2 and cytokeratin 20 mRNA were not expressed by circulating cells from normal subjects. Aldolase A and cytokeratin 19 were thus excluded from subsequent analysis because they were aspecifically expressed by normal circulating cells.

The expression of cytokeratin 20, EPG2 and CEA mRNA species was verified by lung cancer samples. Cytokeratin 20 mRNA was not expressed by any lung cancer sample, and was therefore excluded from subsequent analysis because it cannot identify circulating tumour cells of lung origin; EPG2 mRNA was expressed by all the 27 lung cancer biopsies tested and CEA mRNA by 29/32 (90.6%); thus the study was continued using these two mRNA markers.

Subsequently, blood samples from the 32 lung cancer patients were analysed, immediately before surgery. EPG2 mRNA was absent from the blood of all the 29 lung cancer patients tested. CEA mRNA was detected in 17/32 cases (table 1). Twelve of these 17 positive patients developed distant metastases within 12 months of blood sampling, *i.e.* two patients had metastases at diagnosis, one patient developed metastases within 3 months of the analysis, six patients within 6 months, two patients within 9 months, and, finally, one patient developed metastases within 12 months of the analysis. Three positive patients died and one was lost to follow-up. One positive patient (*i.e.* no. 27) was free of metastases 24 months after diagnosis. In particular, 6/16 (37.5%) stage I–II lung cancer patients from the study were positive for circulating cells expressing CEA at diagnosis; three of these developed metastases within nine months of the analysis.

Twelve of the 15 negative patients were free of metastases 24 months after diagnosis; one negative patient was lost to follow-up and one died 3 months after diagnosis. In one negative patient (*i.e.* no. 28), the lung cancer sample did not express CEA. In particular, 3/13 stage IIIa lung cancer patients were negative for CEA-expressing blood cells at diagnosis. They did not develop metastases in the 24 months after surgery.

All the RNA samples tested (blood cells and lung tissue) were positive for superoxide dismutase and glyceraldehyde 3-phosphate dehydrogenase housekeeping genes. In all experiments, the two negative and the two positive control samples gave the expected results. The results obtained by the two independent operators were concordant except for three RNA samples from lung cancer biopsies that were negative for CEA in one of the two experiments and positive in the other. The three samples were analysed twice more and were positive. Finally, all samples analysed without reverse transcriptase were negative, thus excluding DNA contamination of RNA samples.

Discussion

The present data indicates that cytokeratin 19 is expressed by blood cells of normal subjects (even though primers were used that excluded pseudogene amplification) and so cannot be used to detect circulating micrometastases in human neoplasias. Thus, the current authors confirm the illegitimate transcription of cytokeratin 19 mRNA by normal circulating cells previously reported [5, 9, 14, 23]. Similarly, aldolase A mRNA was detected in circulating cells from healthy subjects, which limits its use in identifying cases of blood micrometastases. With this study the current authors provide the first data about RT-PCR analysis of aldolase A mRNA in normal blood cells. Aldolase A is involved in the glycolytic pathway, so it is not surprising that normal leukocytes express this mRNA species. Cytokeratin 20, CEA and EPG2 are not expressed by circulating cells from normal subjects, at least not at the level of sensitivity achieved using the present authors' techniques (*i.e.* 1 in 100,000 cells, [12]). Most studies, in agreement with the present authors' data, excluded the illegitimate expression of cytokeratin 20, CEA and EPG2 by normal blood cells [7, 9, 11, 17, 21], although a highly sensitive nested PCR revealed CEA expression by normal blood cells in a low percentage of normal subjects due to illegitimate transcription or to false positives [10, 12], and 20% of lung adenocarcinoma can aberrantly express CK20 [15]. Therefore, before the analysis to detect circulating micrometastases, the current authors recommend that a number of blood samples from normal subjects be tested with the RT-PCR procedure used in order to exclude illegitimate transcription. Furthermore, each mRNA sample must be analysed with and without reverse transcriptase to check DNA contamination, and primers must be selected for cDNA amplification by hybridising with two sequential exons of the target gene to exclude amplification of genomic DNA [10, 11]. Lung cancer samples did not express cytokeratin 20; consequently, this marker cannot be used to detect circulating micrometastases in lung cancer patients. Cytokeratin 20 expression has been demonstrated in colon, pancreas and stomach cancers by RT-PCR [7, 20]. It is conceivable that different neoplasias express different levels of cytokeratin 20, and that the level expressed by lung cancer is undetectable with RT-PCR. The current authors' data confirm that EPG2 is expressed by lung cancer samples [17]. However, it was not detected in circulating cells of any of the studied lung cancer patients; possibly, the very low amount of mRNA expressed by each neoplastic cell is below the detection level of the procedure. Therefore, EPG2 does not appear to be a sensitive marker of micrometastatic circulating cells in lung cancer.

This study confirms that most lung cancers express CEA mRNA. The RT-PCR analysis failed to detect CEA [10] in only 5/56 lung cancer biopsies from the present and the previous study [12]; it is feasible that in these cases the biopsies used for molecular analysis did not include neoplastic cells. In any event, the sample taken for cytohistological diagnosis should be checked for the expression of target mRNA species before the mRNA is analysed in blood. CEA expression in circulating cells seems to be related with the development of distant metastases in most lung cancer patients of the present study, who were monitored for 24 months. The test seems to be particularly useful to identify stage I–II lung cancer patients at a higher risk for distant metastases who would benefit little from surgery as demonstrated in three cases from the current study. Similar to the current results, HERDER *et al.* [2] reported that 15% of stage I–II patients developed metastases within 12 months of surgery and thus underwent futile surgery [2]. Furthermore, lung cancer patients with a higher risk of metastatic spreading

could be selected for adjuvant chemotherapy, which does not improve survival in unselected lung cancer patients after surgery [4, 24]. The search for CEA-expressing circulating cells could be associated to instrumental staging procedures to select stage III lung cancer patients eligible for surgery; in fact, three stage III lung cancer patients from the current study did not developed metastases during follow-up. They were negative at the RT-PCR analysis of CEA mRNA in circulating cells at diagnosis.

In conclusion, this prospective study of a novel cohort of lung cancer patients supports and extends the concept that carcinoembryonic antigen messenger ribonucleic acid analysis in circulating cells is a useful aid in identifying lung cancer patients who have a high risk of metastatic spreading. The other messenger ribonucleic acid species do not seem to be useful markers, at least for lung cancer: cytokeratine 19 and aldolase A are not specific signals of neoplasia, being expressed also by normal circulating cells; cytokeratin 20 is not expressed by lung cancer biopsies and thus is not a marker of circulating tumour cells of lung origin; epithelial glycoprotein 2 is not expressed at appreciable levels by circulating cells from neoplastic cancer.

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