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Detection of cell-free nucleic acids in bronchial lavage fluid supernatants from patients with lung cancer

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Abstract

The aim of this study was to determine whether nucleic acids are detectable in cell-free bronchial lavage supernatants, and whether it is possible to find alterations in this DNA and RNA of genes known to be present in lung tumour cells. DNA was isolated from cell-free lavage supernatants from 30 and RNA from 25 lung cancer patients. The DNA was examined for microsatellite alterations (MA) and the RNA analysed for the expression of seven tumour-associated genes. Intact DNA and mRNA could be isolated from all cell-free bronchial lavage supernatants. MA were found in lavage supernatants of 12/30 patients and in lavage cells of 6/30 patients. Altogether alterations were found in 14/30 patients. Analyses of tumour-associated gene expression showed positive results, with at least one marker in the lavage supernatants of all 25 patients. Thus, we could demonstrate, for the first time, that it is possible to isolate intact DNA and RNA from cell-free bronchial lavage supernatants. Their quantity and quality is sufficient for further amplification by polymerase chain reaction (PCR)/reverse transcriptase (RT)-PCR. Altogether, tumour-associated changes were detected in DNA samples from 47% of the patients and in RNA samples from all of the patients analysed.

Keywords: Free nucleic acids; Bronchial lavage; DNA/RNA isolation; Lung cancer; Early detection

1. Introduction

Lung cancer is the leading cause of cancer-related deaths in Western countries [1] and despite massive efforts to develop therapeutic strategies, improvements in survival rates have been modest [2]. Bronchoscopy with endobronchial biopsy is the key diagnostic approach in cases of suspected lung cancer. Bronchial washing is an additional valuable diagnostic tool, especially when tumour manifestations are beyond the visible large airways. There is good evidence that bronchial lavage (=bronchial washing) improves the yield of bronchoscopy with a minimal effort, time and cost being involved [3]. In up to 40%, positive results can be obtained in the evaluation of solitary pulmonary nodules [4]. Nevertheless, conventional cytology is often not conclusive and further invasive procedures are required. Therefore, the establishment of more sensitive diagnostic methods for the detection of lung tumour-associated changes is of the utmost importance.

Molecular alterations precede morphological changes of lung cancer cells and might therefore be useful for the detection of early cancer [5,6]. So far, tumour-associated genetic alterations have been exclusively analysed in tumour cells, or in cells isolated from sputum or bronchial lavages. Several years ago, Stroun and colleagues published their observations that cancer-related changes were also detectable in free circulating plasma DNA [7]. During recent years, tumour-associated alterations like point mutations in tumour suppressors and oncogenes, microsatellite alterations, and gene hypermethylation have been detected in free circulating DNA in the plasma/serum of lung cancer patients [8–10]. Recently, it has also been shown, by us and others, that plasma/serum from tumour patients harbours free circulating

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RNA and that tumour-associated gene expression changes are detectable in this body fluid as well [11–15]. Based on these findings, we examined a set of genes, known to be specifically expressed/overexpressed in lung cancer (such as *hnRNP*-B1 and *PGP-9.5*), and genes whose expression is associated with a variety of different solid tumours (such as the *MAGE* gene family, *HER-2/neu* and the genes coding for the two *telomerase* subunits). In addition, we looked for the expression of genes coding for STK 15/*Aurora*-A/*Aurora*2, that are known to induce centrosome amplification and aneuploidy [16] and *Pericentrin*, which is an integral centrosome protein that plays a role in microtubule organisation [17].

We asked (i) whether it is possible to isolate nucleic acids from cell-free bronchial lavage supernatants which is discarded after the cell harvest, (ii) whether these nucleic acids might orginate from the tumour and be helpful in detecting tumour-associated alterations, and (iii) whether it is possible to find tumour-associated alterations that are not detectable in other compartments.

To answer the first question, we established and optimised several different methods for the isolation of DNA and RNA from cell-free lavage supernatants. In a second step, these nucleic acids were examined for characteristics known to be associated with a malignant lung disease. For the analysis of DNA, we used a set of eight microsatellite alterations (MA) known to occur frequently in lung cancer. The isolated RNA was examined for the presence of mRNA species, coding for genes known to be expressed/overexpressed in lung tumour cells. Thus, the aim of this work was to look for lung tumour-associated alterations in DNA and RNA from cell-free lavage fluid which is obtained during bronchoscopy and is presently discarded after the cell harvest.

2. Materials and methods

2.1. Patients

We analysed samples from 42 unselected patients with a histologically confirmed tumour (36 non-small cell lung cancer (NSCLC) patients, 6 small cell lung cancer (SCLC) patients). 30 patients were used for the DNA-marker analysis, 25 patients for the RNA-marker analysis. The clinical details of the patient population are listed in Table 1.

Bronchoscopy was done under sedation (midazolam, 2–10 mg intravenously (i.v.)) and local anaesthesia (prilocain 1%, 10–30 ml). We used flexible video bronchoscopes (Pentax and Olympus) with a working channel of 2 mm. Lavage was performed at the site of a visible tumour. In cases of peripheral tumour manifestations, the bronchoscopic examination was guided by computed tomography (CT) to the segment most likely to contain the tumour. Warm saline (0.9% NaCl) was injected in 20 ml aliquots through the working channel and harvested by pooling into sterile collection tubes (yield 15–35 ml). The samples were kept refrigerated until they were processed (within 2–4 h). Blood samples were obtained from peripheral veins before the application of sedation or any other medication. The research protocol was approved by the local ethics committee of the University Hospital Charité, Berlin, Germany and written informed consent was obtained from all patients included in this study.

2.2. Processing of blood and lavage samples

Peripheral blood samples (obtained in ethylene diamine tetraacetic acid (EDTA) tubes for plasma) were spun for 10 min at 350*g* at room temperature and the plasma fractions were carefully transferred into new tubes. The cell-free supernatants were re-centrifuged for 15 min at 2000*g* at 4 °C, aliquoted and stored at −80 °C. The cell pellet from the EDTA tubes was diluted with 5 ml 0.9% NaCl solution and the peripheral blood lymphocytes (WBC) fraction obtained by layering on a Ficoll cushion and centrifugation for 30 min at 700*g* at room temperature (rt). The cells were washed with 0.9% NaCl and stored as a dry pellet at −80 °C. The lavage fluid obtained during bronchoscopic examination was spun once (10 min at 350*g* at rt), the cell-free supernatant was removed, aliquoted and stored at −80 °C. The cell pellet from these samples was washed once with 0.9% NaCl solution and frozen as a dry pellet at −80 °C.

2.3. DNA isolation

DNA from WBC and lavage cells was isolated by standard procedures using phenol/chloroform extraction and ethanol precipitation [18]. The DNA from lavage fluid samples was isolated by using either one of two methods. In the first approach, DNA was extracted by mixing the lavage fluid (3–5 ml) with the same volume of Tris-saturated hot phenol (65 °C), and incubation for 30 min at 65 °C. This was followed by centrifugation for 20 min at 3150*g* at 4 °C, transfer of the upper DNA-containing aqueous phase into a new tube and precipitation by adding 1/10th volume of 5 M NaCl and one volume of isopropanol. After leaving the tubes at −80 °C for several hours or overnight the pellet was dissolved in solution D [19] and precipitated again. Finally, the DNA was dissolved in 30 μl 10 mM Tris, pH 8.0.

The alternative method we used involved the use of the QIAamp DNA Blood Mini Kit according to the manufacturer's instructions (Qiagen, Germany), with the following modifications. Lavage fluid volume

Table 1
Clinical data of the study population and bronchoscopic findings

Patient no.	Gender	Age (years)	Smoking (py)	Diagnosis	Tumour	DNA	RNA
1	m	66	20	NSCLC	Visible	√	
2	m	66	75	NSCLC	Visible	√	
3	m	63	50	NSCLC	Visible	√	
4	f	64	15	NSCLC	Visible	√	
5	m	75	75	SCLC	Visible	√	√
6	m	66	20	NSCLC	Visible	√	
7	m	55	20	NSCLC	Not visible	√	√
8	f	62	60	NSCLC	Visible	√	
9	f	58	80	NSCLC	Visible	√	
10	m	72	40	NSCLC	Visible	√	
11	m	65	30	NSCLC	Not visible	√	
12	m	72	30	NSCLC	Visible	√	
13	f	66	20	NSCLC	Not visible	√	
14	m	52	40	NSCLC	Visible	√	√
15	m	69	40	NSCLC	Visible	√	
16	m	47	50	SCLC	Visible	√	
17	f	70	50	NSCLC	Visible	√	√
18	m	66	50	NSCLC	Visible	√	√
19	m	62	40	NSCLC	Not visible	√	√
20	m	60	40	NSCLC	Visible	√	√
21	m	59	40	NSCLC	Visible	√	√
22	m	61	80	NSCLC	Visible	√	√
23	m	45	40	NSCLC	Not visible	√	√
24	m	69	Non-smoker	NSCLC	Visible	√	
25	m	72	40	SCLC	Visible	√	
26	m	70	40	NSCLC	Not visible	√	√
27	m	57	100	NSCLC	Visible	√	√
28	m	60	60	SCLC	Visible	√	
29	f	59	40	SCLC	Visible	√	√
30	m	67	40	SCLC	Visible	√	
31	m	62	50	NSCLC	Not visible		√
32	m	72	30	NSCLC	Not visible		√
33	m	69	30	NSCLC	Not visible		√
34	m	68	45	NSCLC	Not visible		√
35	m	52	50	NSCLC	Visible		√
36	m	66	40	NSCLC	Not visible		√
37	m	65	Non-smoker	NSCLC	Not visible		√
38	m	60	20	NSCLC	Not visible		√
39	f	61	Non-smoker	NSCLC	Visible		√
40	m	65	Non-smoker	NSCLC	Visible		√
41	m	75	40	NSCLC	Visible		√
42	m	62	120	NSCLC	Visible		√

m, male; f, female; py, years ((age at presentation−age started smoking−years stopped)×(cigarettes/day)); NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; DNA, material used for DNA-marker detection; RNA, material used for RNA-marker detection; no., number.

applied to each column was increased to 1 ml. Furthermore, the washing step employing buffer AW2 was performed three times, incubation time with the elution buffer EL was extended to 5 min and the DNA was eluted two times each with 75 μl of the elution buffer that was supplied with the kit.

2.4. Microsatellite analysis

A set of eight microsatellite markers, located on six different chromosomes was used. One of the two polymerase chain reactions (PCR) primers for each marker was end-labelled using the T4 polynucleotide kinase (Invitrogen) and ^{32}P-γATP (3000 Ci/mmol specific activity, Amersham International) and added to the PCR master-mix. The details for the primer sequences and PCR reactions are listed in Table 2. The labelled PCR products were run on a 7% acrylamide gel including 5.6 M urea and 32% formamide. The dried gels were analysed by autoradiography without intensifying screens at rt. The autoradiographs were analysed manually by two investigators independently. MA were defined as the presence of new bands (shifts) in the DNA isolated from lavage fluid or lavage cells compared with DNA from lymphocytes (WBC). A loss of heterozygosity (LOH) was scored when the intensity of

Table 2
Primer sequences for microsatellite analysis and gene expression studies

Marker	Location	Repeat	Primer sequence 5′→3′	Product size (bp)	Annealing temperature (°C)
D9S942	9p21	GA	Forward GCA AGA TTC CAA ACA GTA Reverse CTC ATC CTG CGG AAA CCA TT	98	52
D9S171	9 p21	CA	Forward AAG TGA ACC TCA TCT CTG TCT G Reverse CAA CCC TAG CAC TGA TGG TA	159	66
D16S541	16q12	ATAG	Forward CCA CAC CAG CGG TTT TTC TAA Reverse CAC ACT TTA CAC ACA CCT ATA CCC	160	63
D20S82	20pter	GAAA	Forward TGG GCA ACA GAG TGA GAC C Reverse GCC AGT GAC CTA ACA GAA ATC A	240	50
UT5320	8q24	GAAA	Forward GGA AGG AAG GAA GGA AAG GA Reverse GGA GCT TGA GAT GAC CCT GA	206	58
ACTBP-2	6q13	AAAG	Forward AAT CTG GGC GAC AAG AGT GA Reverse ACT TCT CCC CTA CCG CTA TA	233–323	59
D3S1234	3p21	CA	Forward CCT GTG AGA CAA AGC AAG AC Reverse GAC ATT AGG CAC AGG GCT AA	99–121	60
D3S1300	3p21	CA	Forward CAC ATTC TAG TCA GCC TGA GAC A Reverse AAA AT T GCA CCC CTA CCA CA	133	60
L17686	7q31	AAAG	Forward TCA TAT TAT CTG AGT GTG ATT TGT AGG Reverse TGC ACA ACA CAT ACA CGT TCT C	295	53
β-actin			Forward ACG GCT CCG GCA TGT GCA AG Reverse TGA CGA TGC CGT GCT GCA TG	196	61
hnRNP-B1			Forward TGT TCC TTT GGA GAG GAA AAA G Reverse TTG ATC TTT TGC TTG CAG GA	164	62
PGP 9.5			Forward CTG TGG CAC AAT CGG ACT TA Reverse TGT CAT CTA CCC GAC ATT GG	203	60
MAGE-2			Forward AAG TAG GAC CCG AGG CAC TG Reverse GAA GAG GAA GAA GCG GTC TG	MAGE-2 = 316 MAGE-A2 = 217	55
Her2/neu			Forward AGC CGC GAG CAC CCA AGT Reverse TTG GTG GGC AGG TAG GTG AGT T	147	62
Aurora			Forward GCT GGA GAG CTT AAA ATT GCA G Reverse TTT TGT AGG TCT CTT GGT ATG TG	219	62
Pericentrin			Forward AGC GTG GGA TCT TCA CAA TC Reverse GCA TGT TGC TCA GAC TCA GG	192	60
Telomerase hTR			Forward GAA GGG CGT AGG CGC CGT GCT TTT GC Reverse GTT TGC TCT AGA ATG AAC GGT GGA AGG	111	63
Telomerase hTERT			Forward TGA CAC CTC ACC TCA CCC AC Reverse CAC TGT CTT CCG CAA GTT CAC	95	63

For all polymerase chain reactions (PCR) and reverse transcriptase (RT)-PCR experiments AmpliTaq Gold (Applied Biosystems) and the 10× buffer supplied with the enzyme was used. Conditions: 45 PCR cycles, final Mg^{2+} concentration of 1.5 mM.

one allele was reduced by at least 50% in comparison to the identical allele in the WBC DNA from the same patient. All experiments were repeated at least once.

2.5. RNA isolation

The RNA was isolated from 1 ml of once-frozen cell-free bronchial lavage supernatant with a QIAamp MinElute Virus Vacuum Kit (Qiagen). Frozen samples were thawed on ice. The samples were processed, using the QIAamp MinElute Virus Vacuum Protocol as recommended by the manufacturer (Qiagen, Germany). Total RNA was eluted in 150 μl AVE buffer. The eluate containing the RNA was precipitated by adding 1/10 volume of 3 M sodium acetate, pH 5.2, and ethanol and overnight incubation at −80 °C. The RNA was pelleted at 3900*g* for 30 min at 4 °C in a microfuge, dissolved in 200 μl diethylpyrocarbonate (DEPC) water, treated with RNase-free DNase I (Roche Applied Science), extracted once with phenol/chloroform and precipitated with 3 M sodium acetate (pH 5.2) and ethanol at −80 °C. The total amount of RNA isolated from 1 ml cell-free bronchial lavage fluid was reverse-transcribed and used for the RT-PCR analysis.

2.6. cDNA synthesis and RT-PCR

The total amount of RNA isolated from a 1 ml aliquot of cell-free bronchial lavage fluid was dissolved in 24 μl of DEPC-treated distilled water. cDNA was synthesised with random hexamer primers (300 ng per tube) and Superscript II Reverse Trancriptase (Invitrogen) in a total volume of 40 μl for 1 h at 42 °C. For each RT-PCR reaction, we used the amount of cDNA corresponding to RNA isolated from approximately 75 μl bronchial lavage fluid. AmpliTaq Gold (Applied Bio-

systems) was used in all the RT-PCR experiments. The details for the primers and the RT-PCR conditions are listed in Table 2. The products of the RT-PCR were separated by running through a 2% agarose gel, and staining with ethidium bromide. The gels were then photographed. The authenticity of the RT-PCR products was verified by sequencing. All experiments were repeated at least once.

3. Results

3.1. DNA isolation and quantification

Based on our experiences isolating circulating DNA from plasma/serum samples, we assumed that the DNA concentration in the cell-free lavage supernatants would be as low or even lower. To increase the DNA yield, we therefore started with 3–5 ml lavage supernatant and used the hot phenol method as the isolation procedure. DNA isolated from the first seven lavage supernatants was quantified by spectrophotometry (Table 3). In one sample, the DNA concentration was below the detection limit, whereas in all other samples the concentration varied from 18 up to 193 ng/μl. To check the integrity and purity of the isolated DNA, and to determine the optimal amount of template DNA, all samples isolated from lavage supernatants and lavage cells were amplified by PCR with one or several microsatellite markers (D16S541 and D9S942, respectively). These experiments showed that all preparations contained a sufficient amount of DNA to be amplified by PCR (Table 4). This holds true even when we were not able to measure any DNA spectrophotometrically (sample 11, Table 3).

3.2. Microsatellite alterations in bronchial lavage supernatants

In comparisons with DNA from WBC, we detected MAs (i.e. shifts and/or LOH) in one or more microsatellite markers in 14/30 patients, i.e. 47% (Fig. 1). The majority of alterations were detected in DNA from lavage supernatants (12/30 patients). In contrast, MAs were shown in DNA isolated from bronchial lavage cells in 6/30 tumour patients, and in 4 of them these were the only alterations detectable (Table 4). This observation confirms the pathological finding that only 6 patients had a positive cytology (Table 4). There was a clear difference in the frequency of alterations of the markers used. One group of markers (D20S82, UT5320, ACTBP-2, D3S1234, D3S1300, and L17686) showed 4–6 alterations, whereas in the second group only one alteration was found (D9S942) or no change at all (D9S171).

Table 3
Quantification of DNA isolated from cell-free lavage supernatant

Patient	Diagnosis	Lavage fluid (ml)	DNA yield (μg)
2	NSCLC	4.5	2.2
4	NSCLC	5.5	3.6
5	SCLC	4.0	5.8
6	NSCLC	5.5	3.8
9	NSCLC	5.5	0.33
11	NSCLC	4.5	n.d.
12	NSCLC	4.8	0.54

For quantification, all DNA samples were isolated with the hot phenol method and finally dissolved in 30 μl 10 mM Tris, pH 8.0; n.d., DNA concentration was too low to be quantified by this method.

3.3. Isolation of RNA from lavage supernatants

Since we expected a low RNA concentration in cell-free lavage supernatants, we started with 1 ml lavage fluid for the RNA isolation with the QIAamp MinElute Virus Vacuum Kit instead of the 500 μl recommended by the manufacturer. There was no visible RNA pellet in any of the tubes and therefore no effort was made to quantify the samples. Instead, the total amount of RNA (obtained from 1 ml lavage fluid) was used for each cDNA synthesis. To check the integrity of the isolated RNA and to ensure that there was sufficient cDNA to be amplified, all samples were subjected to RT-PCR with *β-actin*-specific primers. These experiments showed that there was sufficient cDNA in the samples analysed and the *β-actin*-specific RT-PCR gave a positive result with the probes from all 25 patients (Table 5).

3.4. Detection of tumour-associated mRNA in bronchial lavage supernatants

RNA isolated from the lavage fluid of 25 lung cancer patients was then tested for the presence of a panel of genes known to be expressed/overexpressed in lung

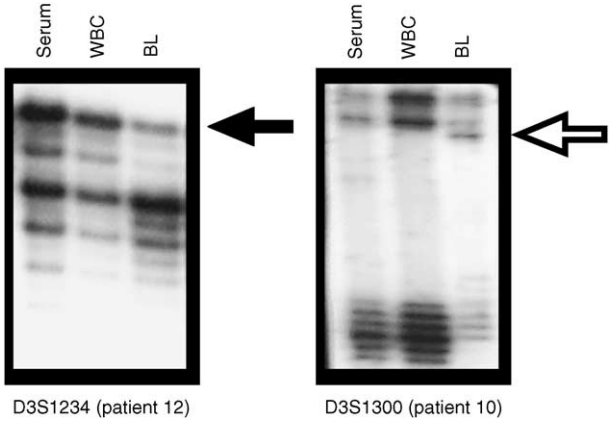

Fig. 1. The autoradiographs shown are representative samples of microsatellite alterations (MA) found in the DNA isolated from cell-free lavage supernatants. Patient 12 showed a LOH in the upper allele of D3S1234 (filled arrow); patient 10 had a shift in the D3S1300 marker (open arrow). WBC, white blood cells; BL, lavage.

Table 4
Microsatellite analysis of DNA isolated from lavage supernatant and lavage cells

Patient	Amplification control		D9S942		D9S171		D20S82		UT5320		ACTBP-2		D3S1234		D3S1300		L17686		Cytology
	Lavage	Cells	Lavage	Cells	Lavage	Cells	Lavage	Cells	Lavage	Cells	Lavage	Cells	Lavage	Cells	Lavage	Cells	Lavage	Cells	Cells
1	√	√	–	–	–	–	–	–	–	–	sh	–	–	–	–	–	–	–	–
2	√	√	L	–	–	–	–	–	–	–	–	–	L	–	L	–	–	–	–
3	√	√	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	√
4	√	√	–	–	–	–	–	–	–	–	L	–	–	–	–	–	–	–	–
5	√	√	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
6	√	√	–	–	–	–	–	–	–	–	–	–	–	L	–	–	–	–	–
7	√	√	–	–	–	–	–	L	–	–	–	–	–	–	–	–	–	–	–
8	√	√	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
9	√	√	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10	√	√	–	–	–	–	–	–	sh	–	sh	–	sh	–	sh–	–	–	–	–
11	√	√	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
12	√	√	–	–	–	–	–	–	–	–	–	–	L	–	–	–	–	–	–
13	√	√	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
14	√	√	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
15	√	√	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	√
16	√	√	–	–	–	–	–	–	L	–	–	–	–	–	–	–	–	–	√
17	√	√	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
18	√	√	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
19	√	√	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
20	√	√	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
21	√	√	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
22	√	√	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
23	√	√	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
24	√	√	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	√
25	√	√	–	–	–	–	–	–	L	–	L	–	–	–	L	–	–	sh	–
26	√	√	–	–	–	–	sh	L	–	–	–	–	–	–	–	–	–	–	–
27	√	√	–	–	–	–	L	–	–	–	–	–	–	–	–	–	–	–	√
28	√	√	–	–	–	–	–	–	–	–	L	–	–	–	–	–	–	–	–
29	√	√	–	–	–	–	L	–	–	–	–	–	–	–	–	–	sh	sh	√
30	√	√	–	–	–	–	–	–	L	–	–	–	L	L	L	L	–	sh	–

Amplification control: the isolated DNA was checked for sufficient quantity and quality and an optimal template amount in PCR with D16S541 and D9S942, respectively. Positive results are marked with √. L, loss of heterozygosity (LOH); sh, shift. Patients with a positive finding at routine cytology are marked with √.

cancer by qualitative RT-PCR. The presence of *hnRNP-B1* coding mRNA was detected in 18 patients, *PGP-9.5*-specific mRNA in 8 patients, *Pericentrin*-specific mRNA in 12 patients, *MAGE-2/A2* mRNA in 20 patients, *HER2/neu* in 6 patients, *Aurora* in 4 patients, *hTR* in 25 patients, and *hTERT* in 3 patients (Table 5, Fig. 2). Altogether, in all 25 patients with a histologically-confirmed tumour, the expression of at least one of the genes examined was found in RNA isolated from cell-free lavage supernatants.

4. Discussion

To test the assumptions that (i) it might be possible to detect cell-free DNA in lavage supernatants, and (ii) to find alterations in this DNA known to be associated with lung cancer, we used two methods for the DNA isolation from cell-free lavage supernatants and were able to obtain sufficient DNA to be amplified by PCR with either method. When the first seven DNA samples isolated from cell-free lavage supernatants were measured, we found in six of them enough DNA to be quantified spectrophotometrically (Table 3). With the set of eight microsatellite markers chosen for the analysis, 30 alterations were found in the samples examined and 14/30 patients had a least one microsatellite alteration. The majority of alterations (22/30) was found in DNA isolated from cell-free lavage supernatants and not from lavage cells. This result is in good agreement with the observation that the detection of morphologically-altered cells was only possible in 6/30 patients by routine cytology (Table 4). In 12/14 patients, in whom microsatellite alterations were observed, this abnormality was detected in DNA from the lavage supernatant exclusively. In contrast, only 2 more patients with microsatellite alterations were identified by analysing the DNA from their lavage cells (Table 4).

All of the microsatellite markers chosen for our set have been shown to be altered in lung cancer. The markers

Table 5
Results of gene expression analysis of lavage supernatant

Patient no.	Amplification control (*β-actin*)	*hnRNP-B1*	*PGP 9.5*	*MAGE-2*	*HER2/neu*	*Aurora*	*Pericentrin*	*Telomerase*	
								hTR	*hTERT*
5	√	√	–	√	–	–	–	√	–
7	√	–	–	√	–	–	√	√	–
13	√	–	–	√	–	–	–	√	–
17	√	√	√	√	–	–	√	√	√
18	√	√	√	√	–	–	√	√	–
19	√	–	–	√	–	–	–	√	–
20	√	√	√	√	–	–	√	√	–
21	√	–	√	√	√	√	–	√	–
22	√	√	–	–	–	–	–	√	–
23	√	–	–	√	–	–	–	√	–
26	√	√	√	√	√	√	√	√	–
27	√	√	√	√	–	–	√	√	–
29	√	–	–	–	–	–	–	√	–
31	√	√	–	√	–	–	–	√	–
32	√	√	–	√	–	–	–	√	–
33	√	√	√	√	√	√	√	√	–
34	√	√	–	–	–	–	–	√	–
35	√	√	√	√	√	√	√	√	√
36	√	√	–	√	–	–	√	√	–
37	√	√	–	√	√	–	–	√	–
38	√	√	–	–	–	–	√	√	–
39	√	√	–	√	–	–	–	√	–
40	√	√	–	√	–	–	√	√	–
41	√	–	–	√	–	–	–	√	√
42	√	√	–	–	√	–	√	√	–

RNA isolated from lavage supernatants was reverse-transcribed with Superscript II (Invitrogen) and the cDNA amplified by RT-PCR. A pair of *β-actin*-specific primers was used to check for the quantity and quality of the isolated RNA. Positive results with this gene were obtained with RNA preparations from all 25 patients. The analyses of tumour-associated gene expression which gave positive results are marked with √.

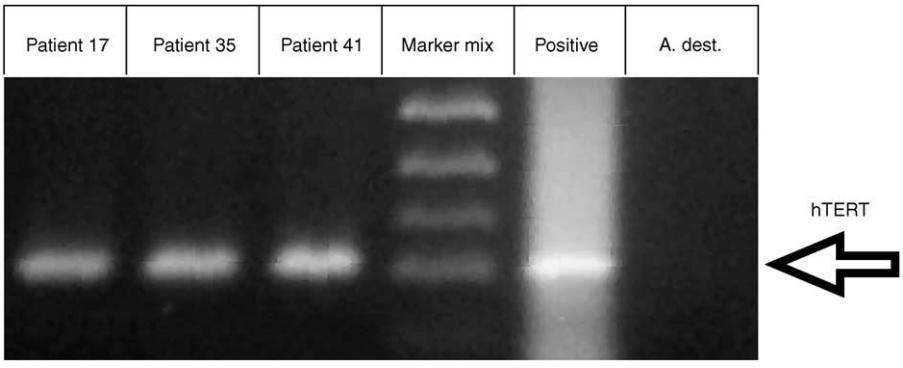

Fig. 2. RT-PCR for the detection of the *hTERT* subunit of the *telomerase* gene from RNA isolated from a cell-free bronchial lavage supernatant. Lanes 1–3: RNA extracted from patients 17, 35 and 41; lane 4: molecular weight marker; lane 5: RNA isolated from a tumour cell line known to be positive for the expression of *hTERT* gene.

D3S1300 and D3S1234 are located on chromosome 3p, a region known to harbour several genes which are important for the development of lung cancer (reviewed in Ref. [20]). In addition, alterations on this chromosome are among the earliest described in preneoplastic lesions [21]. Alterations on chromosome 9p are also very frequent in lung cancer. The marker ACTPB-2 had been used by us and others for the detection of alterations in free circulating serum DNA [9,10]. The marker L17686 was used, since an association between the detection of mutations in the p53 tumour suppressor gene and an increased frequency of alteration in this microsatellite has been demonstrated [22].

The observation that not all microsatellite markers chosen for this analysis work equally well in detecting alterations in DNA from lavage supernatants, confirms results reported by us and others analysing free circulating plasma/serum DNA and DNA from tumour cells

[10,23]. In most of the studies analysing the molecular genetic alterations in free circulating DNA, there was not a complete congruence between the alterations observed in tumour DNA and in free circulating DNA. Possible explanations for this observed discordance are the known tumour heterogeneity regarding its genetic alterations, the existence of tumour subpopulations, their different access to the vascular system, and their different ability to shed DNA into circulation. Therefore, it might be necessary to establish a panel of microsatellite markers for the specific and sensitive analysis of molecular genetic alterations associated with lung cancer in cell-free DNA that is different from a marker panel optimised for the detection of alterations in tumour cell DNA.

Work done in our laboratory and by others has shown that it is possible to isolate intact RNA molecules circulating in the plasma/serum of tumour patients and to detect the expression of tumour-associated genes in this free circulating RNA [11, 12]. Therefore, we expanded our analysis of free nucleic acids and asked whether it is also possible to isolate intact RNA from cell-free lavage supernatants. Starting with an amount of 1 ml lavage supernatant, we successfully isolated enough intact RNA to amplify *β-actin*-specific, reverse-transcribed cDNA in RT-PCR in all 25 samples. The set of tumour-associated genes used for the analysis of free RNA in lavage fluid was chosen for the following reasons:

An overexpression of the *hnRNP-B1* gene and its encoded protein, probably one of the earliest detectable alterations in lung cancer development, was observed in all stage I lung cancer tissues analysed, found in cells of roentgenographically-occult cancers and in bronchial dysplasia [24,25], and in almost all lung cancer subtypes [26]. An overexpression of this protein is highly associated with the development of lung cancer and the detection of its expression in a sputum-based assay was possible 1 year or more prior to the detection of lung cancer by chest X-ray [27]. We showed that it is possible to detect *hnRNP-B1* coding mRNA as free nucleic acid in the plasma/serum of lung tumour patients [12]. Therefore, this gene might be a good candidate for a very specific marker for the early detection of lung cancer. The *PGP-9.5* encoded protein, which removes ubiquitin from ubiquitinated proteins and prevents them from targeted degradation by proteasomes, is highly expressed in human lung cancers and only weakly expressed in the normal lung, which makes it a potential candidate marker for the detection of lung cancer [28]. The melanoma antigen (*MAGE*)-encoding gene family is expressed in various tumour types, including lung, and is thought to be silent in all normal tissues, except testis. An expression of the *MAGE-2/A2* gene is common, not only in NSCLC, but also in bronchial epithelium following severe carcinogen insult [29]. The detection of *HER-2/neu* mRNA expression in 25–35% of NSCLC patients seems to be of biological significance and is associated with an unfavourable prognosis [30]. The expression of both *telomerase* subunits and protein-based activity was observed in most sputum and lavage fluid samples from lung cancer patients [31] and proved to be useful as a sensitive marker for malignancy [32]. The *Aurora* (*STK 15/Aurora-A*) and *Pericentrin* proteins are part of the centrosome structure and a link between the genetic instability observed in most malignant tumours and centrosome defects is assumed [16,17]. When tested in preliminary experiments, we found *Aurora* and *Pericentrin* to be expressed in lung tumour cell lines (data not shown) and included both genes in our analysis. The examination of this set of genes, encompassing a variety of different functions and associated with the development of a solid tumour in general (*telomerase*, *MAGE* gene family, *HER2/neu*, *Aurora*, *Pericentrin*) or lung cancer specifically (*hnRNP-B1*, *PGP-9.5*), might be useful for the sensitive and specific detection of lung cancer at an early stage. In combining the RT-PCR analysis with all the above-mentioned genes, we were able to detect a specific gene expression in the lavage supernatants from all tumour patients. Because of a shortage of material, these experiments could not be repeated with RNA isolated from bronchial lavage cells. The goal of this study was to answer the question of whether it was possible to isolate RNA from cell-free lavage supernatants, and to detect the expression of genes known to play a role in lung cancer development. To answer this question, we used a qualitative RT-PCR assay and did not aim at quantifying these RNAs. This pilot study did not aim at discriminating the patient population from a group of patients with non-malignant lung disease. The results enable us to do further investigations concerning sensitivity and specificity of the alterations found.

To our knowledge, this is the first study aiming at the detection of nucleic acids in cell-free bronchial lavage supernatants. We were able to show that the quantity and quality of the isolated DNA and RNA is sufficient to be amplified in PCR and RT-PCR analyses, respectively, and that it is possible to detect tumour-associated alterations in cell-free DNA and RNA molecules. Currently, the lavage supernatant obtained during bronchoscopy is discarded after the cell harvest. Since this study has shown that it is possible to detect additional molecular genetic alterations in DNA and RNA from cell-free lavage supernatants, not obtained in the analysis of lavage cells, these samples might be useful tools for the detection of tumour-associated alterations in cancer patients.

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References

1. Greenlee RT, Hill-Harmon MB, Murray T, Thun M. Cancer statistics. *CA Cancer J Clin* 2001, **51**, 5–36.
2. Richards MA, Stockton D, Babb P, Coleman MP. How many deaths have been avoided through improvements in cancer survival? *Br Med J* 2000, **320**, 895–898.
3. Schreiber G, McCrory DC. Performance characteristics of different modalities for diagnosis of suspected lung cancer. *Chest* 2003, **123**, 115S–128S.
4. Baaklini WA, Reinoso MA, Gorin AB, Sharafkaneh A, Manian P. Diagnostic yield of fiberoptic bronchoscopy in evaluating solitary pulmonary nodules. *Chest* 2000, **117**, 1049–1054.
5. Mao L, Hruban RH, Boyle JO, Tockman M, Sidransky D. Detection of oncogene mutations in sputum precedes diagnosis of lung cancer. *Cancer Res* 1994, **54**, 1634–1637.
6. Baak JPA, Path FRC, Hermsen MAJA, Meijer G, Schmidt J, Janssen EAM. Genomics and proteomics in cancer. *Eur J Cancer* 2003, **39**, 1199–1215.
7. Stroun M, Anker P, Maurice P, Lyautey J, Lederrey C, Beljanski M. Neoplastic characteristics in the DNA found in the plasma of cancer patients. *Oncology* 1989, **46**, 318–322.
8. Johnson PJ, Lo YM. Plasma nucleic acids in the diagnosis and management of malignant disease. *Clin Chem* 2002, **48**, 1186–1189.
9. Chen XQ, Stroun M, Magnenat JL, *et al*. Microsatellite alterations in the plasma DNA of small cell lung cancer patients. *Nat Med* 1996, **2**, 1033–1035.
10. Bruhn N, Beinert T, Oehm C, Jandrig B, Petersen I, Chen XQ, Fleischhacker M. Detection of microsatellite alterations in the DNA isolated from tumour cells and from plasma DNA of patients with lung cancer. *Ann N Y Acad Sci* 2000, **906**, 72–82.
11. Kopreski MS, Benko FA, Kwak LW, Gocke CD. Detection of tumour messenger RNA in the serum of patients with malignant melanoma. *Clin Cancer Res* 1999, **5**, 1961–1965.
12. Fleischhacker M, Beinert T, Ermitsch M, *et al*. Detection of amplifiable messenger RNA in the serum of patients with lung cancer. *Ann N Y Acad Sci* 2001, **945**, 179–188.
13. Silva JM, Rodriguez R, Garcia JM, *et al*. Detection of epithelial tumour RNA in the plasma of colon cancer patients is associated with advanced stages and circulating tumour cells. *Gut* 2002, **50**, 530–534.
14. Gal S, Fidler C, Lo YM, *et al*. Detection of mammaglobin mRNA in the plasma of breast cancer patients. *Ann N Y Acad Sci* 2001, **945**, 192–194.
15. Chen XQ, Bonnefoi H, Pelte MF, *et al*. Telomerase RNA as a detection marker in the serum of breast cancer patients. *Clin Cancer Res* 2000, **6**, 3823–3826.
16. Zhou H, Kuang J, Zhong L, *et al*. Tumour amplified kinase STK15/BTAK induces centrosome amplification, aneuploidy and transformation. *Nat Genet* 1998, **20**, 189–193.
17. Pihan GA, Purohit A, Wallace J, *et al*. Centrosome defects and genetic instability in malignant tumours. *Cancer Res* 1998, **58**, 3974–3985.
18. Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning, a Laboratory Manual*, 2nd edn. Cold Spring Harbour, Cold Spring Habor Laboratory Press, 1989.
19. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987, **162**, 156–159.
20. Zabarovsky ER, Lerman MI, Minna JD. Tumour suppressor genes on chromosome 3p involved in the pathogenesis of lung and other cancers. *Oncogene* 2002, **21**, 6915–6995.
21. Sanz-Ortega J, Saez MC, *et al*. 3p21, 5q21, and 9p21 allelic deletions are frequently found in normal bronchial cells adjacent to non-small-cell lung cancer, while they are unusual in patients with no evidence of malignancy. *J Pathol* 2001, **195**, 429–434.
22. Ahrendt SA, Decker PA, Doffek K, *et al*. Microsatellite instability at selected tetranucleotide repeats is associated with p53 mutations in non-small cell lung cancer. *Cancer Res* 2000, **60**, 2488–2491.
23. Garcia JM, Silva JM, Dominguez G, Silva J, Bonilla F. Heterogeneous tumour clones as an explanation of discordance between plasma DNA and tumour DNA alterations. *Genes Chromosomes Cancer* 2001, **31**, 300–301.
24. Zhou J, Mulshine JL, Unsworth EJ, *et al*. Purification and characterization of a protein that permits early detection of lung cancer. Identification of heterogeneous nuclear ribonucleoprotein-A2/B1 as the antigen for monoclonal antibody 703D4. *J Biol Chem* 1996, **271**, 10760–10766.
25. Sueoka E, Sueoka N, Goto Y, *et al*. Heterogeneous nuclear ribonucleoprotein B1 as early cancer biomarker for occult cancer of human lungs and bronchial dysplasia. *Cancer Res* 2001, **61**, 1896–1902.
26. Hamasaki M, Kamma H, Wu W, *et al*. Expression of hnRNP B1 in four major histological types of lung cancers. *Anticancer Res* 2001, **21**, 979–984.
27. Mulshine JL, De Luca LM, Dedrick RL, *et al*. Considerations in developing successful, population-based molecular screening and prevention of lung cancer. *Cancer* 2000, **89**, 2465–2467.
28. Hibi K, Liu Q, Beaudry GA, *et al*. Serial analysis of gene expression in non-small cell lung cancer. *Cancer Res* 1998, **58**, 5690–5694.
29. Jang SJ, Soria JC, Wang L, *et al*. Activation of melanoma antigen tumour antigens occurs early in lung carcinogenesis. *Cancer Res* 2001, **61**, 7959–7963.
30. Brabender J, Danenberg KD, Metzger R, *et al*. Epidermal growth factor receptor and HER2-neu mRNA expression in non-small cell lung cancer Is correlated with survival. *Clin Cancer Res* 2001, **7**, 1850–1855.
31. Sen S, Reddy VG, Guleria R, Jain SK, Kapila K, Singh N. *Telomerase*—a potential molecular marker of lung and cervical cancer. *Clin Chem Lab Med* 2002, **40**, 994–1001.
32. Dikmen E, Kara M, Dikmen G, Cakmak H, Dogan P. Detection of *telomerase* activity in bronchial lavage as an adjunct to cytological diagnosis in lung cancer. *Eur J Cardiothorac Surg* 2003, **23**, 194–199.